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A MANUAL

OF

CLINICAL LABORATORY METHODS

BY

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TO THE MEMORY

OF

MY FATHER

ROBERT JAMES CUMMER, M.D.

THIS BOOK IS

AFFECTIONATELY DEDICATED

PREFACE.

In offering this manual for the use of students and practitioners, the object is to present clinical laboratory methods in concise and accessible form. While devoted largely to a description of methods, the underlying principles, the indications for performing tests and the significance of the results are outlined. Accordingly the plan adopted in most chapters has been as follows: (1) Outline of routine examinations; (2) description of the simpler qualitative methods which are frequently employed; (3) description of quantitative methods or those of intricate technic; (4) discussion of findings in various morbid conditions. When several methods are given, usually the preferred one is indicated.

Details are entered into more fully in some cases than in others. For example, the method of counting blood cells has been given meticulously, since it is elementary and its procedures are among the first which must be mastered by the student. It may seem that disproportionate attention has been given to certain subjects, such as the histology and pathology of the blood, but experience in teaching has shown this to be desirable.

It is hoped that both the liberal use of cross references in the body of the text and the synoptic résumés of quantitative procedures in the chapters on Blood and Urine, will facilitate use of the book in the clinical laboratory.

The bibliography, which will be found at the end of the text, contains references to original articles on the newer methods, and will prove helpful to readers desiring to consult the literature. The standard works to which reference is made, have been used freely.

I desire to express my thanks to many of my Cleveland colleagues who read portions of the manuscript and made valuable suggestions, particularly to Drs. Henry L. Sanford, Harvey A. Berkes, J. Edgar McClelland, Harold N. Cole, Richard Dexter and Carl F. Ulrich and to Dr. Henry Schroeder of New York City for his advice regarding the relation of urinary examinations to life insurance. I am deeply indebted to Professor Howard T. Karsner of Cleveland for reading and criticizing the entire manuscript, and to Professor Victor C. Myers of New York City for revising the section on the chemical examination of blood and urine. Mr. John. C. Harding of the Cleveland Medical Library has rendered invaluable assistance in reading proof, arranging the bibliography and verifying references, as did my former secretary, Miss Irene F. Higgins, in preparing the manuscript and correcting proof.

To my wife is due my gratitude for her interest and encouragement, which was helpful in the preparation of even this small volume.

Clyde L. Cummer.

Cleveland, 1922.

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CLINICAL LABORATORY METHODS.

CHAPTER I.

THE EXAMINATION OF THE BLOOD.

HEMOGLOBIN DETERMINATIONS AND ENUMERATION OF CORPUSCLES.

Introductory.—The simplest forms of blood examination are those which have to do with the determination of the relative amount of hemoglobin, the number of red and white corpuscles, and the study of suitably stained films of dried blood. The methods for enumeration of corpuscles require practice and patience, but the necessary technic is soon acquired when the necessary effort is made. These methods should not be attempted at all unless one be willing to spend the time to perform them carefully, since the results of hasty work lead only to fallacious conclusions.

Equipment.—The apparatus needed for an examination of this sort is relatively simple and is included in the list given in the Appendix for the minimum equipment of the clinical laboratory. Apparatus and reagents required for unusual procedures will be indicated with the description of these methods (Figs. 1 and 2).

Indications for Making Blood Examinations.—In practically all clinics, a white cell count is done routinely together with the hemoglobin estimation on admission. If above or in the neighborhood of 10,000 a stained specimen should be examined, and a differential count made. In febrile conditions, in all obscure conditions, and whenever anemia is present, recourse should be had to the hemoglobin determination, enumeration of white cells, and an examination of a stained specimen, with a preliminary differential of at least 150 cells. This does not require much time, indeed the more frequently it is done, the easier it will be to do, and the information will illuminate many diagnostic problems.

In office practice or in dispensary work a determination of hemoglobin should be made almost as a matter of routine. The results may either point to the necessity for further examination along hematological lines or indicate its futility. A percentage below 85 per cent. calls for a count of the red corpuscles, but a figure above this scarcely justifies the labor under ordinary circumstances in the absence of suggestive clinical signs.

Obtaining Blood for Examination.—*For Counting Corpuscles, Hemoglobin Estimations, etc.*—A few drops of blood may be obtained by simple puncture of the skin. A lance with a triangular blade is best adapted for this purpose (Fig. 2, *a*), but in case of emergency a clean steel pen may be utilized by breaking off one nib (Fig. 2, *d*). The instrument may be sterilized by dipping the point in alcohol. For puncture in adults the fleshy part of the ear lobe or the tip of the finger may be used, while with infants and young children it is better to use the great toe or the heel. The skin should be prepared by rubbing it with alcohol. This is wiped dry and the resulting hyperemia is allowed to subside. If the lance be handled with the right hand, the second finger should be held near the tip, as shown in Fig. 16, *a* so that it may serve as a guard against puncturing. After puncture the drop should be obtained with the slightest pressure possible, made a distance from, rather than close to, the puncture, and

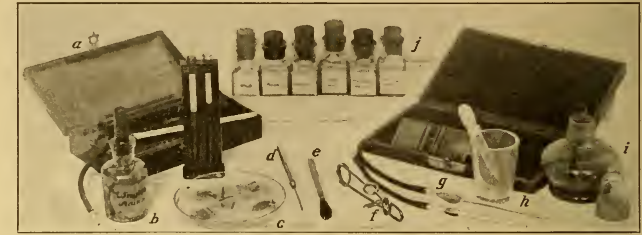

Fig. 1.—Equipment for blood counting. *a*, Sahli hemoglobinometer; *b*, Wright's stain; *c*, clean cover-slips; *d*, blood lancet; *e*, camel's-hair brush; *f*, cover-slip forceps; *g*, hemocytometer (chamber and pipettes); *h*, distilled water and dropper; *i*, alcohol lamp; *j*, one ounce bottles for diluents, strapped together with adhesive.

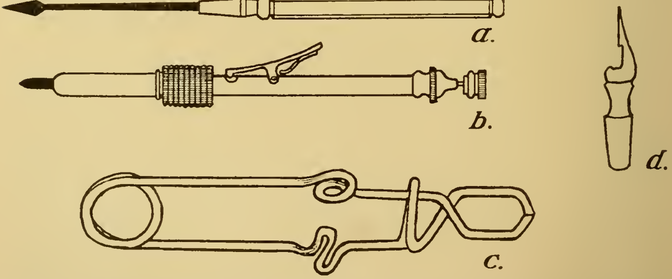

Fig. 2.—Apparatus used in blood counting. *a*, blood lancet, preferred form; *b*, spring lancet; *c*, Stewart cover-slip forceps; *d*, steel pen which can be used for puncturing ear.

care should be taken that the drop is not diluted with tissue juices, as would be the case if the skin were squeezed. The purchase of complicated spring instruments is not desirable (Fig 2, *b*).

Determination of Hemoglobin.—*Comparison of Methods.*—For general utility Sahli's instrument is the method of choice (Fig. 3). When carefully standardized, it is reasonably accurate, sufficiently so for ordinary clinical purposes. The cost is much less than that of the Dare or v. Fleischl instrument. It is available under all circumstances since either daylight or artificial light may be employed. Haldane's method would be preferable were artificial illuminating gas always easily obtained. Both Sahli's and Haldane's methods have the advantage of using as the standard of comparison a solution of hemoglobin derivative. The Sahli method has been criticised by numerous workers. It is undoubtedly inaccurate for scientific purposes, though for ordinary clinical work it is practical and certainly accurate enough, much more so than any other readily available method. Palmer has devised a method which is both simple and accurate, employing carbon monoxide and the Duboscq colorimeter. The disadvantage is the fact that gas is not readily available in some localities, the preparation of carbon monoxide is not always feasible, and the method scarcely lends itself to use except when the patient and the laboratory are in close proximity.

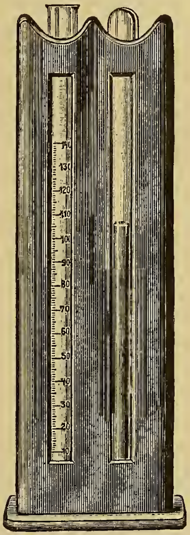

Fig. 3.—Sahli's hemoglobinometer. (Simon.)

Sahli's Method.—First shake the hermetically sealed standard tube containing the solution of acid hematin. Be sure that the graduated tube and pipette are clean and thoroughly dry. With a dropper put decinormal hydrochloric acid ($\frac{N}{10}$ HCl) in the graduated tube to the mark 10. With the pipette draw up blood to the 20 cmm. mark, wiping off any blood on the outside of the pipette. Eject the blood into the acid in the graduated tube and mix by drawing the mixture back and forth in the pipette. Allow this to stand one minute, then add distilled water until the color matches that in the standard tube, holding both tubes in the black frame and viewing them with light transmitted through the ground glass background. The height of the fluid in the graduated tube is read off on the scale and the percentage of hemoglobin is secured by computing according to the equation furnished with each instrument.

The apparatus should be cleaned carefully after use by rinsing the graduated tube and pipette with distilled water, alcohol, and ether.

Before using, be certain that both graduated tube and pipette are absolutely clean and dry.

Renewal of Standard Solution.—When the standard solution deteriorates, as it will do after a period of months, it should be replaced by fresh fluid. We have found the following method a workable one: The end of the tube is broken off after making a deep nick with a sharp file. The tube is then washed thoroughly with distilled water and sterilized by dry heat. Fresh solution is prepared according to the following formula:

Normal human blood	0.5 cc.
$\frac{N}{10}$ hydrochloric acid	5.0 cc.
Distilled water	25.0 cc.
Mix, and add glycerin	25.0 cc.

$\frac{N}{10}$ hydrochloric acid may be obtained which will be sufficiently accurate for this purpose by adding to 11.7 cc. of HCl (C.P., U.S.P. VIII) sufficient distilled water to bring the volume up to 1000 cc.

Blood is obtained by puncturing a vein of a normal adult with a cell count 5,000,000, using a dry sterile Luer syringe. The $\frac{N}{10}$ HCl, distilled water and glycerin are sterilized by boiling separately in a flask and are cooled before mixing. All glassware should be sterilized by dry heat. While the amount of the mixture given above is considerably in excess of what is required for filling one standard tube, the use of larger quantities is conducive to accuracy. When the solution has been prepared, the required amount is placed in the standard tube, which may be sealed by inserting a small sterile cork, dipped in boiling paraffin.

It is possible to secure new tubes (Eimer & Amend Co., New York City, or Steele Glass Co., Philadelphia, Pa.) and to seal them in a flame. We have found that it is difficult to obtain tubes of exactly the same bore as the calibrated tubes, so it is well to make the standard tube furnished with the instrument last as long as possible.

When working with a standard tube prepared in this way, the percentage may be read directly from the graduated tube after the patient's blood has been diluted to match the standard.

Van Slyke's Gasometric Method for Determining the Hemoglobin.—A comparative simple method has been devised by Van Slyke for determining accurately the hemoglobin of the blood by means of the gasometric determination of the oxygen which it is able to absorb. The apparatus which he has described for estimating the carbon dioxide content of the plasma is utilized. The other apparatus required includes a separatory funnel, glass stirring rod, 2 cc. pipette with its lower delivery mark 3 or 4 cm. above the tip, heavy test-tube or cylinder, thermometer and barometer. The necessary reagents are redistilled caprylic alcohol, ammonia solution made by diluting 4 cc. of concentrated ammonia in a liter of distilled water, saponin powder if available and saturated solution of potassium ferricyanide. The latter is freed from oxygen by boiling and is kept in a burette under a layer of paraffin oil 2 to 3 cm. thick.

Procedure.—Three or more cc. of oxalated blood (see page 167) are introduced into a separatory funnel and distributed in a thin layer about the inner wall to permit the maximum contact with the air and to assure complete saturation of hemoglobin with oxygen. The funnel is rotated for a few minutes to keep the blood in a thin layer and the blood is then transferred to a thick test-tube or cylinder.

The blood gas apparatus is prepared by introducing into it five drops of caprylic alcohol and 6 cc. ammonia solution. If saponin powder be available, as much is added to the ammonia as will stick to the end of a glass rod. After the ammonia has been introduced into the 50 cc. chamber of the apparatus, the chamber is evacuated as described elsewhere (page 201) and the air is extracted from the ammonia by shaking the apparatus for about fifteen seconds. The extracted air is expelled and the process of extraction repeated to make sure that no air is left in the solution. Then about 2 cc. of air-free ammonia are forced up into the cup of the apparatus. The aërated blood is stirred with a rod to secure even distribution of the corpuscles and a portion is drawn into the 2 cc. pipette and run under the ammonia in the cup of the apparatus. The blood is now drawn into the 50 cc. chamber, the ammonia being allowed to follow it and to wash it in. An additional drop or two of ammonia may be added from the pipette to complete the washing. The blood and ammonia are thoroughly mixed in the chamber and allowed to stand until laking is completed. When saponin is present, about thirty seconds are required; when not, about five minutes. When complete hemolysis has taken place, 0.4 cc. of the saturated potassium ferricyanide solution is introduced through the cup to set free the combined oxygen. The apparatus is now evacuated by lowering the leveling bulb until only a few drops of mercury remain above the level of the lower stopcock and shaken with a rotary motion to whirl the blood in a thin layer around the wall of the chamber. If the blood were completely laked, this should require about one-half minute. Since water does not absorb oxygen rapidly enough to cause an error, the solution may be left in the 50 cc. chamber during the reading and the reading taken by holding at a sufficient height to balance the column of the water solution. To make certain that all the oxygen was obtained by the first extraction, the apparatus is evacuated again, the blood again shaken, and the reading taken again. If there is an increase the extraction must be repeated.

After each analysis the 50 cc. chamber should be washed out with the ammonia solution to prevent the formation of a black precipitate.

Calculation.—First it is necessary to subtract from the gas measured the volume of air which is dissolved physically by 2 cc. of blood at the prevailing atmospheric pressure and room temperature. The air physically dissolved is given in the second column of the accompanying table. When the observed reading has been corrected by the subtraction of this figure, it is reduced to standard barometric and thermometric conditions by multiplying by $(0.999-0.0046\ t) \times \frac{\text{barometer}}{760}$, t representing the temperature in degrees Centigrade. Van Slyke's table also gives in the third column factors by which the corrected gas volume per cent. or in the fourth column the percentage of hemoglobin. The percentage of hemoglobin is based upon Haldane's standard of a blood having an oxygen carrying capacity of 18.5 per cent., containing approximately 14 gm. hemoglobin per 100 cc.

FACTORS FOR CALCULATING RESULTS FROM ANALYSIS OF 2 CC. OF BLOOD SATURATED WITH AIR.

Temperature.	Air physically dissolved by 2 cc. of blood; subtract from gas volume read in order to obtain corrected gas volume, representing O_2 set free from hemoglobin.	Factor by which corrected gas volume multiplied in order to give:	
		Oxygen chemically bound by 100 cc. of blood.	Per cent. hemoglobin, calculated on the basis of 18.5 per cent. oxygen = 100 per cent. hemoglobin.
C.°	Cc.	Cc.	Per cent.
15	0.037	$46.5 \times \frac{B}{760}$	$251 \times \frac{B}{760}$
16	0.036	46.3 "	250 "
17	0.036	46.0 "	249 "
18	0.035	45.8 "	247 "
19	0.035	45.6 "	246 "
20	0.034	45.4 "	245 "
21	0.033	45.1 "	244 "
22	0.033	44.9 "	242 "
23	0.032	44.7 "	241 "
24	0.032	44.4 "	240 "
25	0.031	44.2 "	239 "
26	0.030	44.0 "	237 "
27	0.030	43.7 "	236 "
28	0.029	43.5 "	235 "
29	0.029	43.3 "	234 "
30	0.028	43.1 "	233 "

Example of calculation:
Observed gas volume. Temperature, 20° C., 750 mm. . . . 0.450 cc.
Correction for dissolved air (see table above) . . . . . . 0.034 cc.
Corrected gas volume, 0.450 minus 0.034.
$0.416 \times 44.8 = 18.65$ volume per cent. oxygen.
$0.416 \times 243.0 = 101.00$ per cent. hemoglobin.

Palmer's Colorimetric Method for the Determination of Hemoglobin.—Palmer criticizes the Sahli method because the standard is not permanent, because there is considerable delay in the development of the full color value, and because it cannot be used for the blood of different species. He has adopted the use of a standard solution of carbon monoxide hemoglobin, preparing a carbon monoxide solution of the blood to be tested and comparing the unknown with the known in a Duboscq or Kober colorimeter.

The apparatus needed includes a good colorimeter, test-tubes 12 x 120 mm., a glass stirring rod, and pipettes calibrated to contain 0.05 cc. and 0.1 cc. The pipettes may be readily prepared from straight glass tubing, since they require no blowing. The point may be rounded off on an emery wheel.

The solutions needed are 0.4 per cent. solution of ammonia, made by diluting 4 cc. of strong ammonia up to 1 liter with distilled water. There must be at hand the standard hemoglobin solution prepared from blood having an oxygen capacity of 18.5 per cent. This may be determined by Van Slyke's gasometric method (see page 20), or when the standard has been properly prepared once, new ones may be made up by standardization against it. The blood is diluted with 0.4 per cent. ammonia solution to make a 20 per cent. solution of blood having an oxygen carrying capacity of 18.5 per cent. This 20 per cent. blood solution is saturated by running illuminating gas through it for ten minutes. Foaming may be prevented by adding a drop of caprylic alcohol. The glass tube through which the gas is passed is withdrawn slowly and the bottle stoppered immediately with a paraffined cork. Rubber stoppers must not be used in connection with hemoglobin solutions. The stopper is sealed with paraffin and kept in the refrigerator protected from light. Palmer states that standards so made have been kept nearly a year with no deterioration. For use in the test a 1 per cent. solution is prepared by adding 95 cc. of 0.4 per cent. ammonia solution to 5 cc. of the 20 per cent. blood solution. This 1 per cent. standard may be made up from time to time and kept in a dark glass bottle or in one painted black. Fresh 1 per cent. solutions should be made up every two or three weeks.

Procedure.—Blood is obtained in the usual manner from the finger or the lobe of the ear. The flow should be free. A 1 per cent. solution is made by drawing 0.05 cc. into the pipette and transferring it to 5 cc. of the 0.4 per cent. ammonia solution in a test-tube 12 x 120 mm. After the blood has been placed into the ammonia solution, some of the mixture is drawn up into the pipette and ejected several times. Ordinary illuminating gas is allowed to bubble through the solution by way of a gas tube for thirty seconds. The solution is now placed in the right hand cup of the colorimeter and the standard 1 per cent. solution placed in the left hand cup. The standard is set at ten and the cup containing the unknown solution is adjusted to it, when the reading is taken. The calculation is $\frac{10}{R} + 100$ equals the per cent. hemoglobin. In the equation, R represents the reading of the scale of the right hand cup containing the unknown solution. If the blood is markedly anemic, 0.10 cc. should be used and the percentage obtained by the calculation divided by two. Venous blood may be used, coagulation being prevented by using a crystal of sodium or potassium oxalate in the syringe which is used for venipuncture (see page 167).

Saturation of the blood by carbon monoxide should be performed under a hood or under a funnel attached to a suction pump. In case illuminating gas is not available, carbon monoxide may be generated by heating oxalic acid with concentrated sulphuric acid and by passing the gas through sodium hydroxide solution to free it from carbon dioxide.

Tallquist Hemoglobinometer.—This consists of a book of absorbent papers and a scale of colors lithographed on paper, the values ranging from 10 to 100 per cent. A drop of blood is absorbed by a bit of absorbent paper furnished in the book and, when superficial moisture has disappeared, the drop is compared with the scale of colors by moving it under the perforations in the color scale. The color which matches is taken as indicating the percentage. The absorbed drop of blood should be large enough to fill the perforations entirely. The method is quite inaccurate, for there is a great variation in the color scales. The scale is especially misleading with anemic bloods, and the information it furnishes for computing the color index in such cases is often worse than useless.

The Miescher-Fleischl Hemoglobinometer.—This standard apparatus gives excellent results but is suitable for laboratory use only on account of its size and the fact that a dark room is required for the readings (Fig. 4). By drawing blood with a special pipette (Fig. 4, *a*) to the 1, $\frac{2}{3}$, or $\frac{1}{2}$ marks dilutions of 1 : 200, 1 : 300, and 1 : 400 respectively are possible. The small lines on either side of the marks represent $\frac{1}{100}$ of the length of the column, so that when the column of blood is not exactly on the desired line, it may be used without adjustment by correcting the result accordingly. A dilution of 200 times is too strong for normal blood, since the reading cannot be transposed to the scale. The blood is diluted with 0.1 per cent. sodium carbonate solution rather than distilled water, since the color matches the scale better. First one side of the deeper cell (Fig. 4, *b*) is filled with distilled water, and then diluted blood is blown out from the pipette into carbonate solution in the other half of the cell. Each side should show a convex meniscus. The glass cover is slid onto the cell carefully so as to leave the two chambers exactly full, and the cap (Fig. 4, *c*) placed over the top to hold it in position and limit the field of vision. The cell is now placed on the stand (Fig. 4, *d*) of the instrument. A yellow light, such as that from a candle or an oil or gas light, is reflected from the mirror (Fig. 4, *e*) so that both halves of the cell are equally illuminated. Sunlight or an electric light must not be employed. Readings are taken by moving the glass prism with the milled head until the color of the prism as seen through the water-filled half of the cell exactly matches that of the diluted blood. The eyes should be rested during the process, as the retina tires rapidly for red, and the scale should be moved by rather wide excursions to both sides, diminishing the excursions until the colors are the same. At least 5 or 6 readings should be made and the average taken. After the blood has been matched in one cell, it may be transferred with the pipette to the cell of lesser depth and readings taken in this. The cells are respectively 15 and 12 mm. high, so that the readings with the lower cell when multiplied by $\frac{5}{4}$ should equal those obtained with the higher. The final readings may be translated to mg. hemoglobin per 100 cc. of blood by means of a scale which accompanies each instrument.

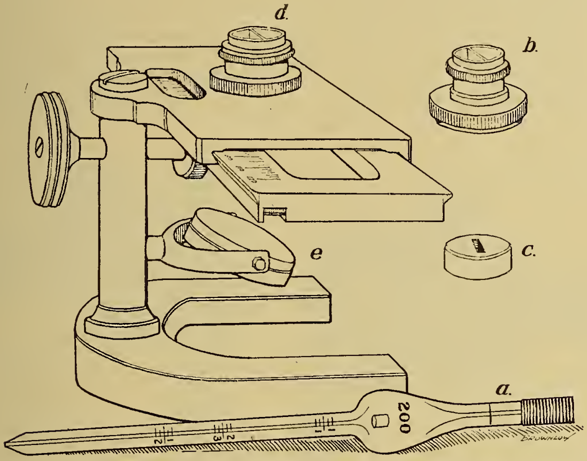

Fig. 4.—The Miescher-Fleischl hemoglobinometer. *a*, pipette; *b*, cell; *c*, cap; *d*, cell in position; *e*, mirror.

The Dare Hemoglobinometer.—The instrument is comparatively costly and easily broken (Fig. 5). There is the additional disadvantage that the color scale is not prepared from hemoglobin derivatives (as it is with the Sahli instrument), but, like the Miescher-Fleischl, is colored glass. Undiluted blood is obtained by allowing it to run into the receiver, formed by two rectangular pieces of glass which are held together in a metal receptacle with a set-screw. The square space should be entirely filled with blood, and the receiver is slipped into its place in the instrument, when it is viewed against a dark background, preferably in a darkened room, with transmitted candle light through the eye-piece. The scale is rotated with the thumb screw until the color of the blood and scale match, when the percentage may be read from the wheel. The instrument has the advantage of a permanent color scale, and gives reliable results in the hands of those accustomed to its use. Electrically illuminated instruments are now obtainable, the bulb is covered with a shield so that it is not necessary to make readings in a dark room.

The Specific Gravity of the Blood.—Some workers employ a determination of the specific gravity of the blood as a means of calculating its hemoglobin content.

Determination of Specific Gravity of the Blood.—In a clean and dry glass urinometer cylinder prepare a mixture of chloroform and benzol in such proportions that the specific gravity will be 1.050 to 1.060. Prick the patient's finger and allow a moderate sized drop of blood to fall directly into the chloroform-benzol mixture. The drop must not be too large, otherwise it will separate and cause confusion. If the drop falls to the bottom of the cylinder, it is apparent that the specific gravity of the mixture is too low, so chloroform should be added drop by drop. When the drop floats on the top, the mixture is too heavy and benzol should be added. In this case add enough benzol to cause the drop to sink to the bottom, then drop in chloroform gradually. When the specific gravity of the mixture is the same as that of the blood so that the drop is suspended near the center of the liquid column, the mixture is filtered and its specific gravity determined by hydrometer.

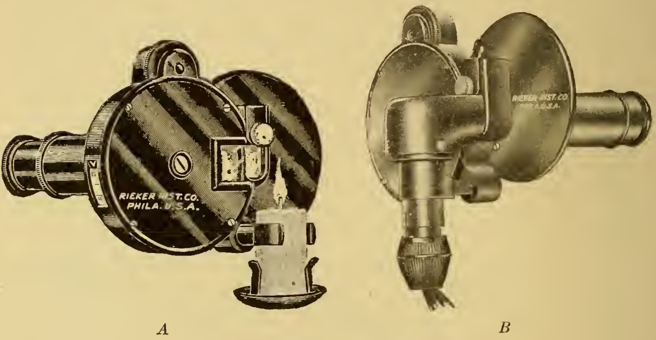

Fig. 5.—Dare's hemoglobinometer.

Hammerschlag's table gives the data require for ascertaining the percentage of hemoglobin from the specific gravity.

Specific gravity.	Hemoglobin.
1.033 to 1.035	25 to 30 per cent.
1.035 to 1.038	30 to 35 "
1.038 to 1.040	35 to 40 "
1.040 to 1.045	40 to 45 "
1.045 to 1.048	45 to 55 "
1.048 to 1.050	55 to 65 "
1.050 to 1.053	65 to 70 "
1.053 to 1.055	70 to 75 "
1.055 to 1.057	75 to 85 "
1.057 to 1.060	85 to 95 "

Findings from Hemoglobin Determinations.—If 100 per cent. is accepted as an arbitrary standard for normal male adults, the percentage with females runs from 90 to 95 per cent. In infancy the amount is much higher as has been shown by the recent work of V. B. Appleton using Van Slyke's gasometric method. The figures are as follow:

Age.	Average, per cent.
1 day	164
2 to 3 days	146
4 to 8 "	135
9 to 13 "	137
2 to 8 weeks	102
3 to 5 months	88
6 to 11 "	87
11 to 23 "	85

In childhood the amount usually reaches 75 to 85 per cent. of the normal adult standard in the sixth year, and gradually increases until the tenth year when it has attained the adult standard.

The variations due to age and sex have been carefully studied by Williamson using the spectrophotometric method of analysis. He has shown that the average hemoglobin content of the adult male is 16.92 gm. per 100 cc. of blood. If this be accepted as representing 100 per cent. on an arbitrary standard and the Sahli hemoglobinometer be standardized accordingly, the findings of Williamson may be translated to terms of this standard according to the scale given in the last column the diagram (Fig. 6) which has been taken from his work. The diagram illustrates graphically the variations due to age and sex.

Enumeration of the Red Blood Corpuscles.—A number of different types of counting chambers are upon the market.[1] We recommend the Bürker type of chamber with Neubauer ruling and prefer the Levy manufacture on account of the clear visibility of the rulings and the general type of construction (Fig. 7). Only standardized outfits should be employed, and it is more than desirable to secure those whose accuracy has been certified to by the United States Bureau of Standards.

The Blood-counting Apparatus.—This consists of two pipettes for diluting the blood, a counting chamber, and a perfectly plane cover-slip. The pipette used for enumeration of red blood cells has ten marks on the stem, the fifth marked 0.5 and the tenth 1. Above the bulb is the mark 101 (Fig. 8). When blood is drawn to the 0.5 mark and diluting fluid to the 101 mark, the dilution is 1 to 200; while it is apparent that with blood drawn to the 1 mark and dilution drawn to 101 mark, the dilution is 1 to 100.

The pipette used for enumerating white cells likewise has ten marks on the stem, the fifth marked 0.5 and the tenth 1, but above the bulb is the mark 11 (Fig. 9). When the blood is drawn to 0.5 mark and dilution fluid to 11 mark, the dilution is 1 to 20; while with blood drawn to the 1 mark and the diluting fluid to the 10 mark, the dilution is 1 to 10.

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[1] An excellent historical review of cell-counting technic will be found in an article by Horace Gray (Am. Jour. Med. Sci., 1921, clxii. 526).

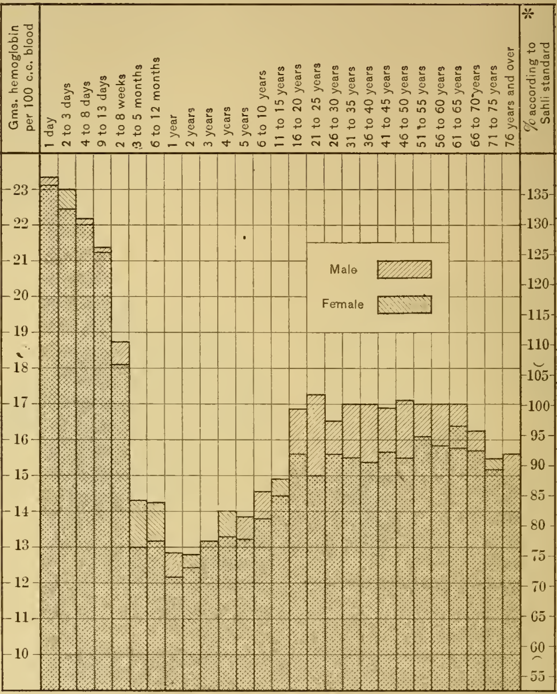

FIG. 6.—Influence of age and sex on hemoglobin values. Diagram, showing average hemoglobin findings at different ages for male and females, according to Williamson's findings.

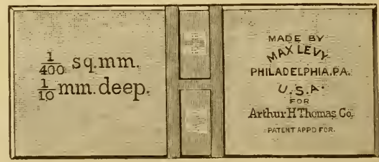

FIG. 7.—Thomas-Levy hemocytometer.

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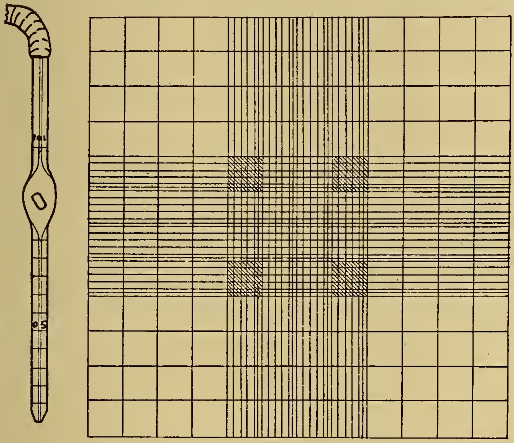
* This column shows values reduced to a percentage basis if the standard is taken as average normal male adult blood, *i. e.*, that containing 16.92 gm. hemoglobin per 100 cc.

The Levy chamber consists of a thick glass slide which is channelled transversely with two deep grooves so that there are two parallel walls on either side of the rectangular platform (Fig. 10). This platform

THE ENUMERATION OF THE RED CELLS.



-SCHEME FOR RECORDING RESULTS-

First Slide.

Upper lt.

5	6	4	3	7
6	5	4	7	5
5	4	5	6	5
4	5	6	4	7
4	3	5	7	5

(127)

Upper rt.

6	5	6	3	2
7	7	4	7	5
5	6	9	4	9
4	8	5	8	3
6	7	4	5	4

(139)

Lower lt.

2	3	5	6	6
6	7	4	5	4
5	7	7	4	7
9	2	8	3	9
4	6	5	8	4

(136)

Lower rt.

4	5	6	8	5
5	5	7	4	7
6	5	4	8	7
6	5	4	7	6
6	6	5	4	8

(143)

Second Slide.

Upper lt.

3	4	5	7	8
7	6	5	6	5
6	8	8	5	7
9	3	7	4	7
5	6	6	7	5

(149)

Upper rt.

6	7	5	5	5
7	7	4	5	7
6	6	5	5	4
4	8	5	8	4
5	6	9	5	8

(146)

Lower lt.

4	4	6	7	6
4	5	5	5	8
5	4	6	5	6
6	6	5	7	6
5	5	5	6	4

(135)

Lower rt.

2	6	5	9	5
3	7	7	3	5
5	4	7	6	8
7	6	6	4	7
6	4	7	9	4

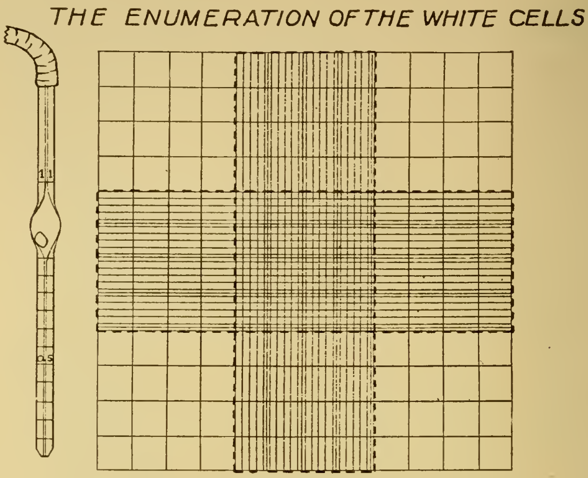
(142)

FIG. 8.—The enumeration of the red cells.

Calculation.—The cells in the corner blocks, each made up of 25 small squares, are counted on two slides. The total contained in 8 corner blocks as shown in the scheme indicated above is 1117. The 8 blocks equal 0.5 square mm., since there are 16 blocks of 25 small squares each in a square mm. Therefore the sum 1117 (contents of 8 blocks) is multiplied by 2 to give the contents of 1 square mm., by 10 to give the contents of 1 cm., since the chamber is only 0.1 mm. deep, and by 200 for the dilution.

Result.—1117 × 2 × 10 × 200 = 4,468,000 cells per cm.

is separated from each of the walls by a depression. The upper surface of the parallel walls is 0.1 mm. above the upper surface of the rectangular platform. The walls have a ground or matt surface, and Newton's rings are not demonstrable as with chambers of older types.



SCHEME FOR RECORDING RESULTS-

First Slide-

	25	
27	22	29
	24	

Total- 127.

Second Slide-

	34	
27	30	22
	29	

Total- 142.

FIG. 9.—The enumeration of the white cells.

Calculation.—The cells are counted in the 5 areas outlined by the heavy dotted lines. The area of each is 1 square mm. Two preparations should be counted, giving a total of 10 square mm. The sum of the contents of the 10 square mm. will give the number of cells in 10 square mm., each being 0.1 mm., or in one cubic mm.

The accurately ground and highly polished cover-glass should bear at one corner the word "UP" or the Bureau of Standards stamp. It should be mounted so that the side engraved "UP" or the stamp is on the upper side. In making a count the cover-glass is placed in position on the parallel walls in such a manner that it rests upon them and does not touch the slide proper.

In the *older types of instrument* the counting chamber consisted of a thick glass slide at the center of which is cemented a small circular platform (Fig. 11). The surface of the platform is subdivided by small rulings, which will be described later. Around the platform is a "moat" or "ditch," separating it from a surrounding "wall" of glass, whose inner border is circular and whose outer border is rectangular.

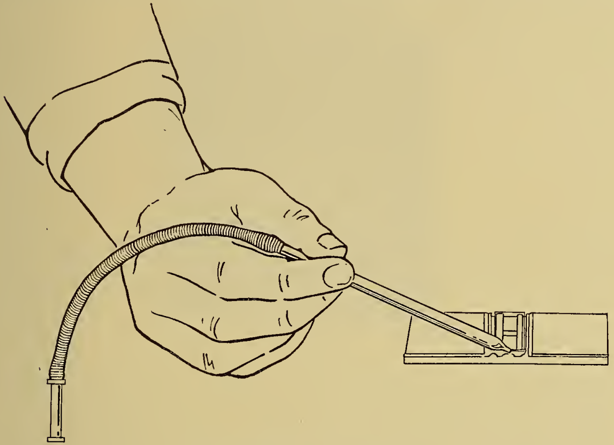


Fig. 10.—Filling the Levy chamber.

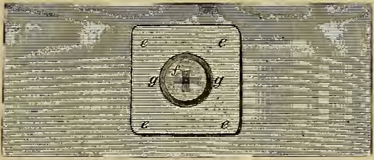


Fig. 11.—Original type of blood counting chamber. *e*,, surrounding "wall" of glass; *f*, circular "platform," surface 0.1 mm. below that of surrounding wall; *g*, circular ditch of moat. (Simon.)

The height of the surrounding "wall" is exactly $\frac{1}{10}$ mm. greater than the height of the central platform or disc, so that when the cover-slip is placed on the "wall," there is a space between the cover-slip and the circular platform just $\frac{1}{10}$ mm. deep.

The simplest form of ruling is found in the *Thoma pattern* (Fig. 12). Here will be found on the surface of the circular platform twenty columns running horizontally and twenty running vertically, each $\frac{1}{20}$ mm. wide. It is evident that when a drop of fluid which has been placed on the platform is covered by a cover-slip, the capacity of the space formed by each tiny square as a base is $\frac{1}{400}$ cmm. The first sixth, eleventh, and sixteenth horizontal and vertical columns are subdivided by a median line. This additional line simply serves as a guide or landmark. The total area covered by the Thoma ruling is 1 square mm.

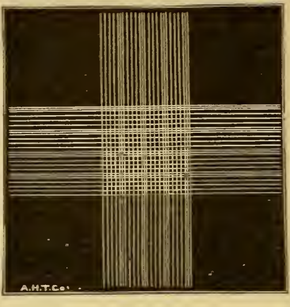


Fig. 12.—Ruling of Thoma chamber.

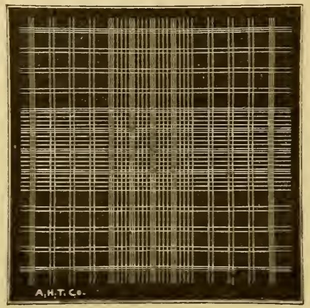


Fig. 13.—Türck chamber.

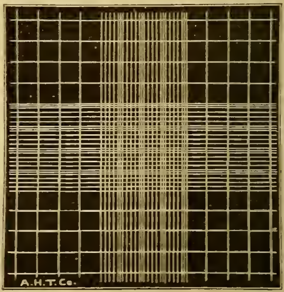


Fig. 14.—Neubauer chamber.

The Türck and Neubauer rulings are in reality elaborations of the Thoma type, the ruled area in both instances covering 9 square mm. instead of 1 square mm. The tiny squares of $\frac{1}{400}$ cmm. area are found in the central square millimeter only. The details of these rulings can be best understood by reference to the cuts (Figs. 13 and 14).

The Bass ruling (Fig. 15) is extremely simple and is less confusing to follow than any of the others since much unnecessary ruling is eliminated. Here too the small squares are $\frac{1}{400}$ cmm. square.

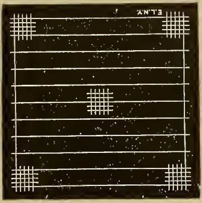


FIG. 15.—Bass counting chamber.

The Enumeration of the Red Blood Cells.—Hayem's solution is a convenient diluent since it keeps well and molds do not grow in it with readiness. The red cells are readily shaken with it to form a uniform suspension, and it is easy to clean the chamber after its use. This is in contrast to Toison's solution, much favored by some, for molds grow in it readily and the glycerin which it contains renders cleaning difficult. The addition of dye to Toison's to stain the white blood cells is not an advantage of consequence, since the number of white cells encountered in making a red count, even if they were not differentiated, would introduce a noteworthy error in the red count only when the white count were exceptionally high. Even Hayem's solution, however, should be filtered occasionally to remove possible molds, since spores might be mistaken for red cells.

The formula for Hayem's solution is:

Mercuric chloride	0.5 gm.
Sodium chloride	1.0 gm.
Sodium sulphate	5.0 gm.
Distilled water	200.0 cc.

The formula for Toison's solution is:

Methyl violet, 5B	0.025 gm.
Sodium chloride	1.000 gm.
Sodium sulphate	8.000 gm.
Glycerol	30.000 cc.
Distilled water to make	160.000 cc.

Procedure: Obtaining Blood and Making the Dilution; Precautions.—1. See that the pipette is thoroughly clean and dry and be sure the glass bead in the bulb of the pipette may be shaken about freely.

2. Have ready for use the pipette with the mark 101.

Obtain a drop of blood according to the previous directions.

Hold the pipette almost horizontally and with gentle suction draw blood to the mark 0.5 (Fig. 16, *b*) wiping off blood on the outside of the

3

pipette with a piece of gauze. If blood is drawn much above the 0.5 mark, bring it to the 6 mark and correct count by multiplying by $\frac{5}{6}$. Should the column of blood rise only a slight distance above the 0.5 mark, draw it back by touching the tip of the pipette with a piece of

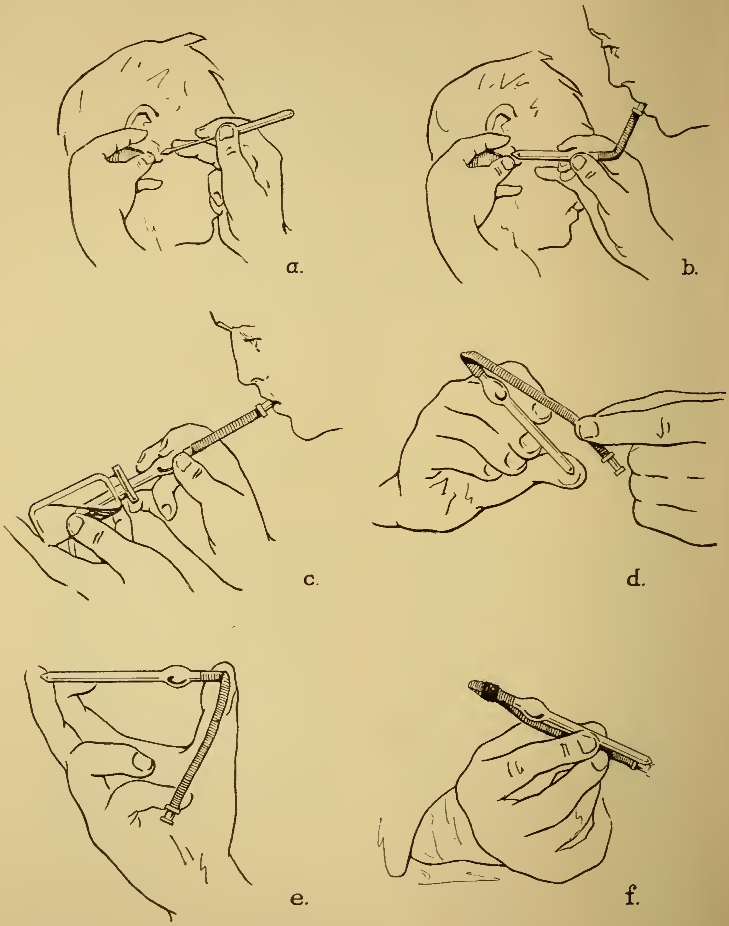


FIG. 16.—Steps in blood counting. *a*, puncturing ear; *b*, obtaining blood with pipette; *c*, drawing up diluting fluid; *e*, shaking pipette; *d* and *f*, preparing pipette for transportation.

gauze. Now draw up Hayem's fluid to the mark 101, meanwhile rotating the pipette to keep the glass bead in motion, holding it in an almost horizontal position (Fig. 16, *c*). When the pipette has been filled, put the thumb over one end of the pipette and the finger over the other and shake vigorously (Fig. 16, *d* and *e*).

If it is desired to take the pipette to another place to make the count (*e. g.*, from the patient's home to the physician's office), place the thumb over the tip of the pipette to seal it, pull the capillary rubber tube down to close off the upper end of the pipette (Fig. 16, *e*) and bind the rubber tubing to the upper portion of the pipette by means of a small rubber band (Fig. 16, *f*). The finger now may be removed from the tip without any danger of the fluid escaping. This will prevent the fluid from running out if the pipette is carried to the laboratory in a horizontal position.

Cleaning the Pipettes.—Great care must be exercised in cleaning the pipettes. Immediately after use the fluid should be ejected. They should be filled with water and emptied, then treated successively in the same way with alcohol and with ether. The last bit of ether should be inhaled, since if it be blown out the moisture of the breath will gain access to the pipette. When the cleaning has been well done, the bead will be shaken about readily and no moisture will be apparent. The cleaning process may be shortened by drawing the various cleaning fluids into the pipettes through the tip, and by detaching the rubber tubing, connecting it to the tip and then expelling the fluid through the larger or upper end of the pipette. The use of a vacuum pump aids materially in reducing the labor attendant upon cleaning. Occasionally blood coagulates in the pipette, due to slow handling or to drawing up alcohol instead of the diluting fluids (a favorite slip with students). A bristle or fine wire will have to be run into the lumen with much care, and when a passage has been cleared, 10 per cent. sodium or potassium hydrate may be drawn in. After standing over night, the pipette can usually be cleaned with numerous changes of the same alkali.

Counting.—When ready to count, clean the chamber and the glass cover-slip thoroughly. Never employ xylol, alcohol, ether, or hot water on the chamber as both the "wall" and "platform" of the old type of chamber are cemented to the glass slide and are readily loosened by these solvents, and even in the new chamber the central ruled rectangular piece of glass is cemented in. Use only gauze, a clean handkerchief or Japanese lens paper with either cold water or the moisture of the breath. It is difficult to get rid of bits of lint, unless one uses a camel's-hair brush. It is absolutely necessary to have no grains of dust, grit or lint on the "wall" or cover-slip. Any foreign body in this place, by raising the cover-slip, would increase the distance between the cover-slip and the circular platform and so increase the number of corpuscles.

When using the old circular type of chamber, it is the writer's practice to clean the slide and cover-slip as directed here and then place the cover-slip on the slide in the position that it will occupy when the preparation is completed. Newton's rings should be looked for. They should appear as concentric, vari-colored rings between the cover-slip and the "wall" of the chamber when the slightest pressure is made on the upper surface of the cover-slip. Their presence indicates that the two highly polished surfaces are in perfect apposition. When any particles of lint or foreign substance come between the cover-glass and the "wall" it will be impossible to demonstrate them. The cover-slip is left in place until the pipette has been shaken, when it is lifted only long enough to put the drop in place. After shaking the pipette, blow out about $\frac{1}{3}$ of the contents of the pipette onto a piece of gauze or blotting paper, and then place a drop on the center of the circular platform.

The size of the drop to be placed on the circular platform can be learned only after repeated trials. With the cover-glass in position, it must be large enough to cover practically all of the platform, but it must not run into the moat. The cover-glass should be placed in position at once. It should not be dropped on carelessly, since in this way bubbles will be included, but with one edge resting firmly in a diagonal position across the "wall," braced against the thumbs, the opposite edge is lowered gently into position with the forefingers. If the drop appears to be satisfactory, ascertain whether Newton's rings be present. If not, the preparation must be discarded.

A preparation should not be counted unless it fulfils the following requirements:

1. It should cover all or practically all over the circular platform.
2. It must not overflow into the moat.
3. No bubbles should be included in the drop.
4. Newton's rings must be demonstrated.

The use of the Levy chamber saves a great deal of time, since the construction is such that cleaning is much easier, and the chance for the cement between the parts to loosen is not so great as with the old circular chamber, the whole slide with the exception of the ruled platform being of one piece of glass. This reduces the possibilities of inaccuracies appearing with deterioration.

In making a count with the Levy chamber, clean both the slides and the ground cover-glass thoroughly, and place the cover in the proper position. Shake the pipette vigorously, eject about $\frac{1}{3}$ of its contents, and by holding it in an almost horizontal though slightly tilted position, bring the tip to the edge of the rectangular platform so that the diluted blood may flow between the platform and the superimposed coverslip (Fig. 10). Stop the flow as soon as the platform has been covered and do not allow the fluid to run into the transverse channels on either side.

After preparing a satisfactory drop, it should be allowed to stand for a few moments, so that the corpuscles may settle. The area for counting should be located with IV ocular and the three objective (Leitz), or 10 ocular and 16 mm. objective (Bausch & Lomb) and then the count should be made with the same ocular and with the 6 objective (Leitz) or 4 mm. objective (Bausch & Lomb). The microscopic field should be darkened by dropping the Abbé condenser slightly and by partially closing the iris diaphragm. In this way both the engraved lines and the blood cells will be accentuated. Our method of counting is to enumerate the number of cells in the four corner blocks, each block containing 25 small squares. One such block is contained in the field when a magnification of 430 times is used (*e. g.*, 10 eye piece and 4 mm. objective of a Bausch & Lomb). This is to be repeated on a second preparation. We have found that counting is much simplified for students if they prepare a diagram of the blocks to be counted and note in each square on the diagram the number of cells seen in the corresponding square of the chamber (Fig. 8). The total number of cells in each block of 25 squares is ascertained. The total of cells in 8 blocks of 25 squares each is multiplied by 2 to find the number per square mm., then by 10 to find the number per cubic mm., since the chamber is only $\frac{1}{10}$ mm. deep, and finally by 100 or 200 according to the dilution which was made.

Example.—First preparation:

Upper right hand block	139	
Upper left hand block	127	
Lower right hand block	143	
Lower left hand block	136	
Total	——	545

Second preparation:

Upper right hand block	146	
Upper left hand block	149	
Lower right hand block	142	
Lower left hand block	135	
Total	——	572

Total for eight blocks of 25 squares each	1,117
Cells per square millimeter	2,234
Multiply by 10 for the depth of the chamber	22,340
Multiply by 200 since blood was drawn to 0.5 mark in pipette	4,468,000

Criterion for Judging Accuracy in Counting.—The difference between the largest and the least number of cells found in two blocks of 25 small squares should not exceed 25. A greater difference is evidence that the preparations are not evenly spread. In class work, it is our custom to insist that students repeat their counts upon their own blood until they secure counts upon successive days which check within 300,000.

Normal Findings.—In healthy male adults the average number of red blood cells is usually accepted as being 5,000,000, though it may range from 4,500,000 to 5,500,000. For a healthy woman 4,500,000 is considered normal after the inception of the menstrual cycle. These figures are not to be accepted too rigidly. Emerson has shown that among medical students the counts ranged from 4,500,000 to 6,700,000. Our own experience with large numbers of apparently normal medical students has been similar. It is not at all unusual to secure counts, carefully made and checked, running over 5,000,000 up to 6,000,000.

In considering counts, the factor of age as well as of sex must be borne in mind. In infants immediately after birth the count may run as high as 7,000,000, but is usually about 6,740,000 (Stengel & White), the average in the nursing period being about 5,580,000 (Wood). The count gradually decreases until about the tenth year, reaching a minimum of 5,100,000 at or about this time. From that period it tends to increase gradually in men until the age of thirty. After forty, the count is apt to drop slowly in men and rise in women (Emerson).

Physiological Variations in the Red Count.—*Temperature* alters the count, the count tending to run higher in winter than in summer. A count shows a transitory increase after a cold bath. The latter is probably due to vasoconstriction. Emerson states that *change of residence* from temperate to tropical regions may cause a diminution of from 500,000 to 2,000,000 cells. There is a rise in the count with *ascent to higher altitudes.*

Variations in Disease.—*By oligocythemia* is meant a diminution in the number of red blood cells per cubic millimeter. It is seen in *pernicious anemia, chlorosis, the leukemias, the secondary anemias,* under which heading is included a variety of inciting causes which will be considered more fully.

By polycythemia is meant an increase in the number of red blood cells per cubic millimeter. A *transient polycythemia* is seen after the *loss of body fluid* in sufficient amount to cause concentration of the plasma, as may occur in severe diarrhea or persistent vomiting. In permanent polycythemias, there is evidently an actual increase in the production of new red cells, since the presence of nucleated red blood cells and polychromatophilia point to active erythrocyte formation. Among the causes of *permanent polycythemia* are *high altitudes, heart disease,* including mitral valve disease, adherent pericardium, and especially congenital heart disease, *lung disease,* particularly empyema and pneumonia, and *protracted poisoning* stimulating the bone-marrow, as by phosphorus and acetanilide. Polycythemia is seen also in a disorder whose etiology is undetermined, referred to as *erythremia,* Vaquez's disease, Osler's disease, etc., characterized by great increase in the number of red blood cells, and of the total blood volume, by cyanosis, retinal changes, and enlargement of the spleen. The increased number of red cells is probably due to hyperplasia of the erythrogenic tissue of the bone-marrow comparable to the condition in myeloid leukemia, where the myelocytic tissue is hyperplastic.

Enumeration of White Blood Cells.—0.5 per cent. acetic acid is used as a diluent, since this lakes the red blood cells and increases the definition of the nuclei of the white cells. Dilute acetic acid may be prepared for this purpose by adding 5 drops of glacial acetic acid to 30 cc. of distilled water. It should be freshly prepared.

The blood is obtained as usual (see page 18). A large drop will be needed. Draw this to the mark 0.5 and then fill the pipette to the mark 11, with dilute acetic acid. Unusual care will have to be taken to make accurate dilutions with the white pipette. The preparation on the counting chamber is made in the manner already described under "Enumeration of Red Blood Corpuscles."

This may be done with the IV ocular and the $\frac{1}{3}$ objective (Leitz), or with the 10 ocular and 16 mm. objective of a Bausch & Lomb, the latter giving a magnification of 87 diameters. This magnification is well fitted to include the 1 square mm. area. The number of cells in an entire square mm. are determined (Fig. 9). In using the Türck, Zappert-Ewing or Neubauer rulings, at least 5 square mm. should be counted on each of two separate preparations and the average taken.

Computation.—The average number of cells per square mm. is multiplied by 10, since the chamber is only $\frac{1}{10}$ mm. deep, and then by 20, since the dilution was 1 : 20 (Fig. 9).

Example.—Suppose that the average number of cells per square mm. is 37.25, then multiply by 10 for the depth and 20 for the dilution, the result would be 7450 per cmm.

Normal Findings.—The normal count for adults ranges between 5000 and 10,000 cells per cubic mm. When the count is below 5000 we say that a *leukopenia* is present, and if above 10,000, there is said to be a *leukocytosis* or *hyperleukocytosis.*

In infancy and childhood the count is considerably higher. During the first few days of life it may reach 20,000 while in the nursing period, the cells average about 12,000. The number steadily diminishes during childhood until about the sixth year, when it may range from 5400 to 12,400 (Wood), conforming to the adult standard at the fifteenth year.

Physiological Variations.—A *physiological leukocytosis* may be seen *during digestion, after exercise, during pregnancy,* and *after cold baths.* These factors should be borne in mind, and when possible it is well to make a blood count before meals. The pathological variations will be considered fully after the consideration of the various forms of leukocytes.

Enumeration of the Platelets.—*Wright and Kinnicutt's Method.*—Solutions required:

I. One to 300 aqueous solution of brilliant cresyl blue. This is fairly permanent if kept in the refrigerator.

II. One to 1400 aqueous solution of potassium cyanide. This must be freshly prepared.

Immediately before use, 2 parts of solution I are mixed with 3 parts of II, and the resulting mixture should be filtered.

A very thin cover-glass is necessary. Special ones are made and may be obtained from microscope manufacturers in which the body of the slip is of usual thickness while the center has been ground out leaving a depression with a very thin floor.

Procedure.—Blood should be drawn to the 1.0 mark and stain to 101 to make a 1 to 100 dilution.

After filling the pipette, preparations may be made in the chamber in the usual way. These should be allowed to stand about ten minutes to permit the platelets to settle completely.

The same method of counting may be employed as in the enumeration of the red cells. The platelets are seen as small round or elongated lilac stained oval bodies. The red cells are decolorized shadows and the white cells are easily distinguished because of the dark blue nuclei. One hundred small squares may be counted, though where greater accuracy is desired, 200 small squares should be counted on each of two fillings of the chamber.

Method of Buckman and Hallisey.—This method has been proposed on account of the difficulty in securing brilliant cresyl blue and to make possible obtaining specimens of blood in a manner which prevents the initial coagulation and consequent thrombin formation with coalesence and metamorphosis of platelets.

The diluting fluid is prepared by dissolving 6 gm. of glucose and 0.4 gm. of sodium citrate in 100 cc. of distilled water. The resulting fluid is filtered and to it is added 0.02 gm. of toluene red, dimethyldiamidotoluphenazin ("neutral red"). Solution is slow but eventually complete. A decigram of crystal violet is added. The mixture is heated to 60° C. and held at that temperature for five minutes when it is allowed to cool at room temperature. After centrifugalizing for ten minutes at 2000 revolutions, the supernatant fluid is filtered twice, each time through three thicknesses of dry No. 30 Whatman filter paper. The solution keeps indefinitely after the addition of 0.2 cc. formaldehyd solution.

Blood is obtained by venipuncture, using a paraffined 18 gauge hypodermic needle, attacked to an evenly paraffined miniature transfusion tube of the type of Lee and Vincent. This is simply a small test-tube open at the top with lower end drawn to a capillary opening which fits the needle. The capacity should be about 6 cc. As the blood wells up into the tube, the tip of the pipette used for counting red cells is introduced and blood is drawn up to the 0.5 mark when the pipette is filled immediately with diluting fluid. After shaking three minutes the counting chamber (Türck or Neubauer) is filled. On this preparation, a red count may be made in three minutes, a white count in five minutes (count the contents of 9 square millimeters) and the platelets in twenty minutes. For the platelets, the high dry objective is used and 4 square millimeters are counted. A second preparation should be counted and the average taken.

The authors state the counts may be made as long as four hours after taking the specimen. In carefully prepared specimens, the platelets are seen as discrete, uniformly distributed, pale blue, ovoid bodies, from $\frac{1}{6}$ to $\frac{1}{2}$ the size of normal erythrocytes, with finely granular cytoplasm and slightly irregular periphery. The platelet count with this method is slightly higher than is obtained by other methods. The red and white counts check very well.

Findings.—Wright and Kinnicutt have found that with their method the number per cmm. ranges from 224,000 to 330,000, the average being 273,250. Normal individuals showed remarkable constancy in the counts.

The findings obtained by Wright agree in a general way with those previously obtained by Hayem and also by Pratt. In secondary anemia, the count is usually high; in pernicious anemia it is generally low. Wright found that in secondary anemia the count was never below normal. The highest counts were above 1,000,000 in cases of anemia due to hemorrhage. These findings are explained by the hyperplasia of the bone-marrow in secondary anemia and the consequent increase in the megakaryocytes, which produce platelets. Similarly the dimunition in pernicious anemia is explained by the fact that though the red marrow is greater in amount, it does not contain as many giant cells as normal. The count was found to be normal in two cases of chlorosis. In lymphatic leukemia it is low due presumably to the lymphomatous formation of bone-marrow and the consequent reduction in giant cells, while in myelocytic leukemia, counts are high, ranging from 600,000 to 860,000.

Early in typhoid fever the counts are normal or below, becoming high during convalescence. In lobar pneumonia they are low, normal, or slightly below normal, increasing rapidly after the crisis. There is no relationship between the platelet count and the leukocyte count. Duke feels that the reason for the variation in the count in infectious diseases is that small amounts of toxin stimulate and large amounts poison the bone-marrow. In two cases of hemophilia, Minot and Lee found platelet counts of from 290,000 to 400,000. They advanced the theory that the normal number of platelets were unusually resistant and therefore unable to liberate a suitable amount of prothrombin.

The Color Index.—The color index is an expression of the hemoglobin content of the red blood cells as compared with the normal. It is determined by dividing the percentage of hemoglobin by the percentage or erythrocytes and may be graphically represented by the fraction:

$$\frac{\text{per cent. Hb}}{\text{per cent. RBCs}}$$

Of course, the normal color index is one. In computing percentages, 5,000,000 is taken as the normal red count and 100 as the normal hemoglobin percentage.

Example.—The color index for a patient whose red count is 4,000,000 and whose hemoglobin percentage was 60 per cent. would be derived as follows:

Four million red blood cells is 80 per cent. of normal.
The hemoglobin is 60 per cent. of normal.
The color index is

$$\frac{\text{per cent. Hb}}{\text{per cent. RBCs}} = \frac{60}{80} = 0.75$$

The color index is of much practical importance in the consideration of the anemias.

The Volume of the Red Cells.—By the use of a rapid centrifuge and of especially prepared pipettes the relative volume of the red cells in a given specimen of blood may be determined. The instrument usually employed is the hematocrit of Daland (Fig. 17). The apparatus consists of two arms which constitute a metal frame, the frame being placed on the end of the centrifuge spindle at right angles to its axis. Each arm of the spindle carries a glass tube, the outer end of the glass tube fitting into a cup-like depression in the frame, the bottom of which is padded with rubber. The inner ends of the tubes fit into a similar surface which is fastened to a spring which serves to hold the tube firmly. Each tube is 50 mm. in length and has a lumen of 0.5 mm. The tube is marked off with a scale of 100 divisions.

To make determinations, the blood is secured by puncture of ear lobe or finger in the usual manner and drawn into the tube by using a rubber connection and mouth piece exactly as for the hemocytometer. The glass tube must be thoroughly cleaned and dry. The operator's

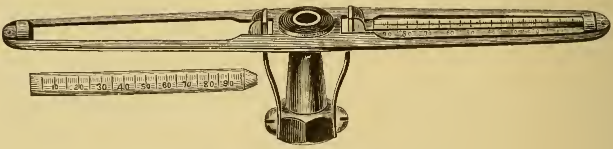


Fig. 17.—Daland's hematocrit. (Simon.)

greased finger is placed over the tip of the instrument while the rubber mouth piece is slipped off, and the glass tube is placed in its place in the frame, adjusting carefully the rubber. The other tube should be filled with water and placed in the opposite side. The rubber pads should be greased with vaseline. Speed is essential to prevent clotting of the blood, so that all preparations should be made in advance. The centrifuge is run for three minutes at a speed of about 10,000 revolutions per minute to ensure complete separation of serum from cells. The leukocytes will be found in a thin white layer at the centripetal end of the tube and usually occupy about one division of the scale. The red cells occupy about one-half of the tube, the proportion being slightly greater for normal males and slightly less for females. While the method serves to give an approximation of the number of erythrocytes in many cases, it is open to criticism in cases of severe anemia, because of the variation in the size of the red cells in the cases, and in leukemia, since the large bulk of leukocytes renders an exact reading difficult.

The chief use made of the method at present is in the determination of the volume-index of Capps.

The Volume-index of Capps.—This method has been devised by Capps to determine the average volume of the erythrocyte. A careful count of the red cells is made with a standardized hemocytometer. The blood volume is determined with the Daland hematocrit. The percentage number of red cells is determined by multiplying the number of million of red cells per cmm. by 20, and the percentage volume of the red cells is determined by multiplying the reading on the scale of the hematocrit by 2. The volume-index is the quotient obtained when the percentage volume is divided by the percentage number. For example, suppose that the column in the hematocrit reaches the mark 45. Then the percentage volume is 90. The red cells number four million so that the percentage number is 80. The volume index is then 90 divided by 80, which is 1.12.

Capps has shown that the cell-volume, as expressed by the volume index, is increased in pernicious anemia, usually more so than the hemoglobin content of the cell. Moderate cases of secondary anemia show only slight loss in cell volume, but in severe anemias, the cells are quite small. In chlorotics the cell volume is diminished as well as the hemoglobin, but is not reduced to such an extent. Capps feels that the volume index is of prognostic significance, stating that patients with a normal or nearly normal volume index recover quickly, while those with a small volume-index respond to treatment slowly.

THE MORPHOLOGY OF THE BLOOD.

The study of the structural details of the various forms of blood cells affords valuable information. It may be accomplished by the preparation of extremely thin films of fresh blood on slides or cover-slips, which may be dried, fixed, and suitably stained.

Preparation of Blood Films.—Films may be prepared for staining by spreading a drop of blood on a slide or between two cover-slips. A good film is absolutely essential for a satisfactory examination and so much may be learned from a stained film that the very considerable amount of time which must be spent in acquiring technic is well spent. Slide preparations are comparatively simple to make. However, they are open to certain serious objections, the chief of which is that when a drop is spread over so large an area one must cover a large portion of the film to obtain a really representative count, since usually the leukocytes are grouped at certain parts of the slide, ordinarily at the edges and the ends. A reliable count therefore, requires more time than does a count made with a cover-slip preparation.

Cover-glass Preparations.—To obtain good films, the following points must be observed faithfully:

(*a*) The cover-glasses must be of the proper size and thickness. They should be at least 22 mm. square and should be of No. 1 thickness. Before using they should be carefully inspected for imperfections and slips showing flaws rejected. Smaller cover-slips are too difficult to handle and thicker ones will not permit focusing with the average oil-immersion lens.

(*b*) They must be absolutely clean and dry and free from dust and lint. Unless this requirement be met, considerable labor will be spent in vain.

(*c*) The drop of blood must be of the proper size, which can be learned only by experience.

(*d*) All operations must be carried out rapidly to avoid coagulation.

(*e*) Constant practice is decidedly helpful in retaining the "knack" of making acceptable films.

Cleaning the Cover-slips.—A number of cover-slips can be cleaned by immersing them in chemically pure nitric acid in a covered glass dish. After standing fifteen minutes, the acid is poured off and the slips are covered very cautiously with 95 per cent. alcohol. A glass plate or cover should be held over the dish since gas will be evolved due to the chemical interaction between the residual nitric acid and the alcohol. After five minutes' time, the alcohol is decanted and the covers are rinsed in repeated changes of distilled water until acid cannot be detected by litmus. The covers may be kept in 95 per cent. alcohol in a covered jar until needed when they may be removed, preferably with clean, unstained forceps, to be wiped with clean gauze. They should not be dried until needed, since dirt and grease will accumulate upon them.

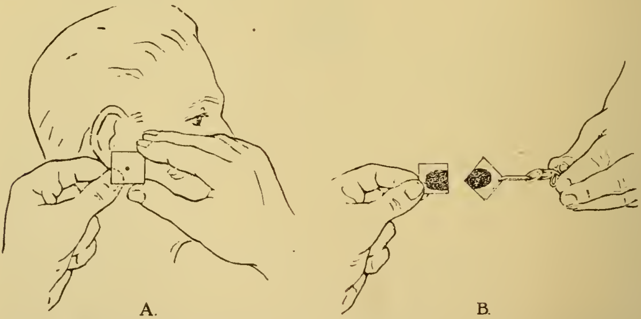


Fig. 18.—Making films on cover-slips. *A*, securing drop on one slip; *B*, pulling slips apart.

Making Cover-slip Preparations.—When the worker is ready to make films, one cover-slip should be gripped in the wire forceps by the corner and placed on the table. The ear or finger should be pricked and a drop of blood obtained (Fig. 16, *a*). Then a second cover-slip is dusted with a camel's-hair brush, passed quickly through the flame of an alcohol lamp or Bunsen burner to drive off the moisture, picked up carefully by the edge, and applied to the drop of blood in such manner that it will pick up the drop, which should be placed in the center of the cover-slip (Fig. 18, *a*). The ear must not be touched with the glass, since oil or dirt adhere. The second cover-slip previously placed in the forceps is grasped quickly, dusted with the camel's-hair brush, and warmed gently in the flame, when the slip bearing the drop of blood is dropped on it gently in such manner that the drop of blood is between the two cover-slips. The slip bearing the blood should be dropped on the first in such manner that the two slips together look like an eight pointed star (Fig. 19, *a*). The drop of blood is allowed to spread until the blood just ceases to flow between the slips when the upper one is pulled away with a single quick stroke, (Fig. 18, *b*), keeping the planes of the two slips parallel and the line of traction of both hands in parallel planes.

A satisfactory film should be tongue-shaped, should cover at least 60 or 75 per cent. of the total area of the cover-slip (Fig. 19, *b*), and should be thin enough to dry almost instantaneously. On examination with the low power of the microscope, the cells through the greater portion of the film should be well separated, not touching each other, and with normal blood the cell outlines should be circular. A film occupying only a small portion of the slip (Fig. 19, *c*) is not acceptable, since there are not enough cells for study.

Preparation of Smears on Slides.—The slides should be thoroughly cleaned with soap and water, rinsed in hot water, and finally in alcohol, when they may be rubbed dry with a clean piece of gauze or preferably a clean old linen or silk handkerchief.

A drop of blood of moderate size is obtained and the end of a slide is touched to the drop in such a way that the drop will occupy a position in the middle of one end. A second slide is now placed over the drop with one narrow end in apposition with the upper surface of the first slide, holding the first slide horizontal and the second at an angle of 30 to 45 degrees with it (Fig. 20, *A*). The blood situated within this acute angle should be allowed to spread along the angle by capillarity when the upper side should be pushed along the first slide so as to drag the drop with it, spreading it on the first slide (Fig. 20, *B*). The position of the fingers in holding the slide is of importance, since the slides must be held so that the upper or "spreader" slide may be pushed practically to the end of the slide on which the smear is being made, while firm support is given. The posture of the hands is shown in the illustration Fig. 20. If the angle between the two slides is more acute than is specified the film will be too thin and the cells will be spread over too great an area.

With a slide preparation it is practically certain that some areas will be fit for study, and the comparative ease of preparation is a distinct advantage to the busy practitioner. There is no objection to the method in hunting for malarial or other parasites, but it is open to the very distinct objection, already voiced, that the leukocytes are unevenly distributed. With a satisfactory preparation, the film should occupy

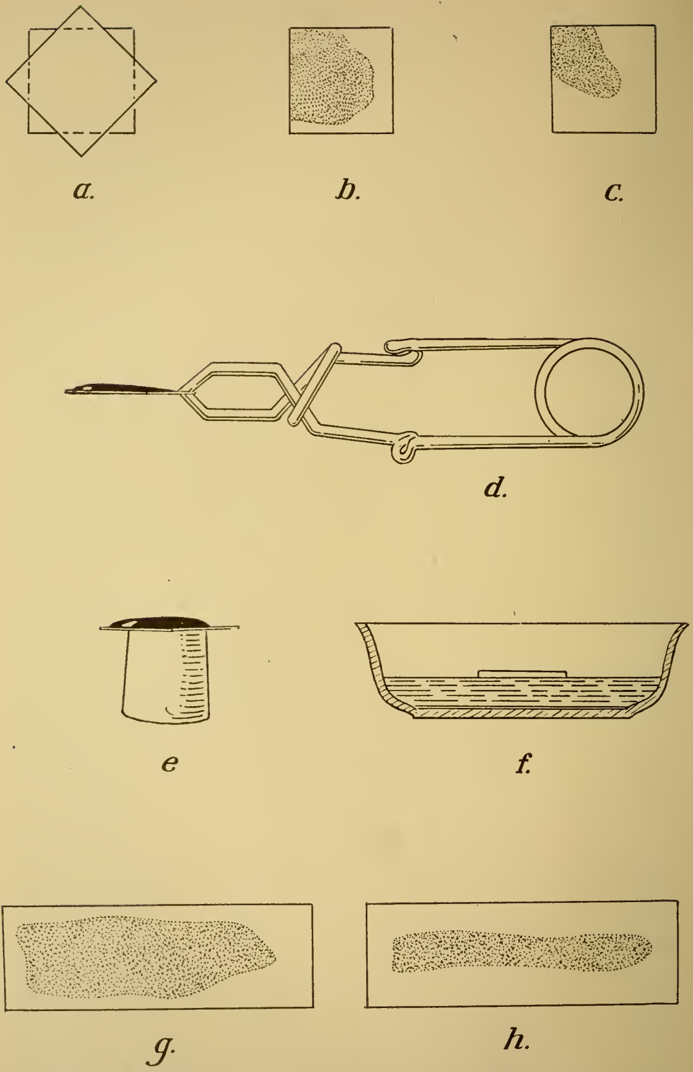


Fig. 19.—Making and staining blood films. *a*, proper position of two cover-slips when drop of blood is between them; *b*, well-distributed film; *c*, poorly distributed film; *d*, staining cover-slip, held in forceps; *e*, mounted on cork; *f*, floating on stain; *g*, well-distributed film on slide; *h*, poorly distributed film on slide.

a broad area across the slide (Fig. 19, *g*) and should not be a mere streak down the center of the slide (Fig. 19, *h*).

Staining.—Practically the only stains used for clinical work are modifications of Romanowsky's. The results obtained by Leishman's, Hastings', and Wright's are practically identical. For clinical work, Wright's stain is excellent. Methyl alcohol serves as a solvent for the stain and as a fixative, so that preliminary fixation by heat is unnecessary. There are a multitude of stains recommended for blood work, but it is vastly better to employ one satisfactory stain, to learn all that one can about it, to become conversant with the possible technical errors and so be able both to guard against them intelligently and to interpret the findings correctly, than it is to attempt to employ two or three stains. The course of wisdom for one who does only a limited amount of blood work would be the complete mastery of a single good method.

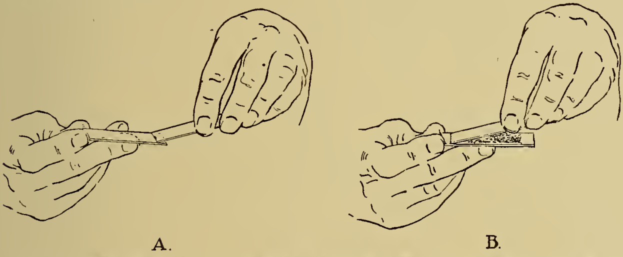


FIG. 20.—Making films on cover-slips. *A*, initial position, allowing drop of blood to spread in acute angle formed by two slides; *B*, final position of upper slide—spread the drop as a thin film on lower slide.

General Considerations Regarding Blood Stains.—The stains of the Romanowsky group employ a mixture of methylene blue and eosin. The specific staining, however, is not due to the methylene blue, but rather to an oxidation product, methylene azure. This is formed in the manufacture of Wright's stain by treatment with heat at 100° C. in the presence of an alkali (sodium bicarbonate). It is probable that several other by-products are formed, including methylene violet. The addition of eosin, a dibasic acid, to methylene blue, a base, results in the formation of the eosinate of methylene blue. The resulting stain therefore contains a complexity of substances, the eosinate of methylene blue, methylene azure, methylene violet, and possibly other dyes. Wilson states that his stain which is prepared by oxidation by silver nitrate as well as by sodium bicarbonate, contains seven compounds, *i. e.*, eosinate of methylene blue, methylene azure, methylene violet, the eosinates of thionin, thionolin, and thionol, and methylene orange.

The preparation of Wright's stain from methylene blue and eosin is given elsewhere in this book (Appendix). If desired it may be secured in powdered form so that it is only necessary to dissolve small quantities in methyl alcohol. This is the most practicable way to prepare the stain. The fluid so prepared keeps for a considerable period of time when the container is tightly corked. The writer has had good working preparations which have given good results at the end of a year, adding a little methyl alcohol from time to time to compensate for loss by evaporation. Making a satisfactory solution in this way is extremely simple, and if contamination with acid or water be scrupulously avoided, the results are usually very satisfactory.

The stain as marketed in the liquid form is very much more expensive and in many instances is none too satisfactory, failing to stain the nuclear elements.

Procedure for Staining.—Precautions.—It is essential that distilled water which is used for dilution, the cover-slip forceps, and the pipette used for adding the water be absolutely free from acid, since the least trace of acid will result in pale, almost colorless, preparations in which it is evident that only the eosin has acted. The points of the cover-slip forceps should be wiped quite dry. The precautions seem childishly elementary, but it is extremely difficult to make students respect them, with the result that complaints are made about the poor bottle of stain which was their lot. Investigation practically always shows that the fault is in the lack of adherence to these simple directions. Further, water must never be allowed to get into a bottle of stain. If the stain is kept in a dropping bottle with a pipette, this pipette must be used for undiluted Wright's only and for nothing else. If the stain is kept in a bottle with an ordinary stopper, stain should be removed from it only with a *dry* acid-free pipette.

The film is grasped in the cover-slip forceps, right side up (Fig. 19, *d*), or placed on a small object, such as a cork or upright test-tube (Fig. 19, *e*). The film is covered with Wright's stain. Staining may be carried out by floating the cover-slip on stain in a staining dish, film side down. After a lapse of one and a half minutes, during which time fixation has been accomplished by the methyl alcohol, acid-free distilled water is added until a metallic film or scum appears on the top of the mixture. About equal quantities of water and stain are required. This stain is now left for about three minutes. When intense staining is desired or when the stain is old and acts slowly, it may be left considerably longer. It is better to err slightly on the side of overstaining, since the intensity may be reduced by free washing. The film is now washed until it is pink, and the result is viewed with the low power lens, according to the standard which follows. If it proves satisfactory, it is blotted very gently, dried thoroughly by holding in the flame, and mounted on a slide in balsam.

Should one wish to preserve the stained blood for a long period of time, the balsam used for mounting should be neutralized. Powdered sodium bicarbonate may be added to Canada balsam which has been thinned to watery consistency by the addition of xylol. The sodium bicarbonate is stirred in well with a glass rod and set aside in a corked wide mouth glass bottle. At the end of twenty-four hours, or when the sodium bicarbonate has settled to the bottom of the bottle, the supernatant clear balsam may be decanted and filtered through a folded filter paper, previously moistened with xylol.

The Criteria of a Satisfactory Stain.—With a satisfactory stain, the red blood cells should be pink or copper colored without any overcasting of blue. This does not refer to an occasional red cell with blue tinge, for this may be due to polychromatophilia, but rather to the discoloration noted throughout an entire film or large portion of it. When seen, it usually indicates overstaining. If generally blue, the preparation is washed again in tap-water until the red cells have assumed the proper color. The nuclei of all leukocytes should be dark blue, the exact shade varying with the particular form of leukocyte. If they are pale blue, the film has not been stained sufficiently. It should be thoroughly dried and the staining process repeated. It is usually just as well to start anew with a fresh film, leaving the stain for a longer period of time. There is of course the possibility that the stain has deteriorated or that either it or the wash water has been contaminated with acid.

Requisites for a Satisfactory Stain.—1. The cytoplasm of the polymorphonuclear neutrophilic leukocytes should show light violet or lilac granules against a pinkish background. If the granules are of an entirely pink tint, the film has been washed too long. While it may be studied in this condition, the eosinophilic and neutrophilic granules will have to be differentiated on the size of the granules. The slip may be restained, employing of course less vigorous washing.

2. The cytoplasm of the lymphocytes should be clear robin's-egg blue. Occasionally it is dark blue, so dark that differentiation of the cytoplasm from the nucleus is extremely difficult. This is due to overstaining. When the defect is seen, the film should be washed again. If the defect cannot be remedied in this way, a new film should be stained.

3. The cytoplasm of the large mononuclears varies from very pale blue to robin's-egg blue. Greater intensity of color betokens the error of overstaining already mentioned.

4. There should be no granules of precipitated stain generally scattered over the entire preparation covering all cells and particularly noticeable over the red cells. This will give rise to much confusion. It is usually due to allowing the staining mixture to stand too long, permitting partial evaporation and a precipitation of stain. This fault would cause much confusion so that a slide presenting it should be discarded and a new one stained with more care.

5. There should be no stain on the reverse side of the cover-slip. When present it may be easily removed by rubbing with a little filter paper moistened with alcohol, but before doing this the slip must be laid film side down on a flat surface to avoid breaking it.

Promptness in staining is rewarded by the best results. Specimens which have been kept unstained for some time require overstaining to bring out the leukocytes properly. As a result, the red cells will take the basic stain too intensely. This defect may be remedied partially by rapidly plunging the stained film in extremely dilute acetic acid (1 drop of glacial acetic acid in 500 cc. of distilled water) and promptly washing in distilled water.

Ehrlich's Triple Stain.—At the present time this stain is used very little for clinical work, since it is difficult to prepare and use, and does not stain malaria parasites or the basophilic granules in mast-cells. It is described here for historical reasons only.

The stain is prepared from saturated aqueous solutions of methyl green, acid fuchsin and orange G. The colors must be chemically pure and should be added in the order and proportions indicated in the formula given by Morris:

Orange G., saturated solution	13.0 cc.
Acid fuchsin, saturated solution	7.0 cc.
Water	15.0 cc.
Methyl green, saturated solution	17.5 cc.
Alcohol	10.0 cc.
Glycerol	10.0 cc.

The solutions are measured in the same graduated cylinder, which should be rinsed between fluids, and the constituents placed in a flask which is shaken after each addition. The mixture should be neither shaken nor filtered. Blood films must be fixed by heat. A long triangular piece of copper about ⅛ inch thick is supported on an iron tripod and the pointed end heated with a Bunsen burner about fifteen minutes until it has reached a constant temperature. The boiling-point is determined by dropping water on the surface and a point selected about an inch further from the flame than the place where drops assume a spheroid shape. The smear is placed on the plate film side down. Periods of time ranging from two minutes to an hour have been recommended.

Fixed films are stained from three to twenty minutes. There is little danger of overstaining. The films are washed in water, dried, and mounted in the usual way. With this stain, red blood cells are buff colored, the nuclei of the leukocytes green, the neutrophile granules lilac, the eosinophile granules crimson, while normoblastic nuclei are almost black.

The Study of the Stained Preparation.—The film should be studied first with the low objective. This is necessary for a general survey, and should always precede a more minute examination. Many hints are obtained with this long distance perspective, as it were, which would be lost if a close-up view alone were depended upon. The distribution, the relative number of red and white cells, and the apparent scarcity of red cells may be readily seen in this way. Then the high dry objective should be used. If a system giving a magnification of about

500 be employed, it is ordinarily unnecessary to employ the oil-immersion lens for routine counts unless it is desired to work out certain finer details, such as the morphology of malarial parasites.

In making a study, it is desirable to observe the points which follow. In submitting reports negative as well as positive findings always should be given in detail so that any one who reads the report may have a word-picture of the film. For one's own satisfaction it is desirable to follow a definite routine.

Points to be Noted in Routine Study of Stained Specimens of Blood.—1. *Distribution of the Red Cells.*—Are they well and uniformly distributed through the body of the slide?

2. *Apparent Number of Red Cells.*—Roughly speaking, are as many seen per field as is customary?

3. *Apparent Number of White Cells.*—Again speaking approximately, are as many seen per field as is customary? It will be well for the student or beginner to determine the average number of leukocytes in each field of a film of the average thickness when examined with a magnification of 500 times.

4. *Relative Proportion of White Blood Cells to Red Blood Cells.*—Are they apparently normal, increased or diminished?

5. *Minute Study of the Red Cells,* including the following points:

(*a*) Is the central pale area normal, increased or diminished?

(*b*) Are the red cells of uniform size or is there anisocytosis?

(*c*) Are the red cells of normal shape, or is there evidence of poikilocytosis?

(*d*) Have the red cells stained uniformly or is there evidence of polychromatophilia?

(*e*) Is punctate basophilia seen?

(*f*) Are nucleated red cells seen? If so, what forms? They should be counted while one makes the differential count, but should not be included with leukocytes. For example, in reporting, one would say that in counting 300 leukocytes, 5 nucleated red cells were encountered; 2 were normoblasts and 3 were megaloblasts.

6. *Blood Platelets.*—Are they apparently present in normal numbers, increased, or diminished?

7. *Parasites.*—If present, detailed study should be made, and an exact diagnosis made. Failing in the latter, detailed drawings should be made so that they may be submitted to others for opinion together with the slide.

The Differential Count.—At the very least 250 white cells should be enumerated. If it be not the intention to cover the entire film in the count, a definite system should be followed so some areas will be taken and studied in all portions of the film in order that the count may be representative.

A mechanical stage is of great assistance, but as an expedient one may use a slide clamped to the stage firmly with a microscope clip. The slide bearing the blood film may be pushed along this as a guide until one column across the film has been studied. Then the guiding slide may be moved sufficiently to bring a new field into view and the film may be moved back. The process may be repeated until the required number of cells has been seen.

In making a differential count, it is useful to employ the following form which follows the classification at present favored in practically all clinics. In reporting to others, the total number of leukocytes counted should be stated, as well as the proportion of each form.

Polymorphonuclears, neutrophilic	(P.M.N.)
Lymphocytes (large and small)	(L.)
Polymorphonuclears, eosinophilic	(E.)
Polymorphonuclears, basophilic (mast-cells)	(B.)
Transitionals	(T.)
Myelocytes, non-granular	(My.-ng.)
Myelocytes, neutrophilic	(My.-n.)
Myelocytes, eosinophilic	(My.-e.)
Myelocytes, basophilic	(My.-b.)

Nucleated red blood cells are not leukocytes and while it is desirable to note them, if any are seen, of course they are not enumerated as leukocytes in the differential.

When specimens are mounted in balsam, one occasionally sees a refractile circle which appears to be within the red cells and is concentric with the cells. In many cases this is due to inclusion of moisture resultant upon insufficient drying of the cover-slip. The cover-slip and the slide with a drop of balsam should be warmed gently over the flame before they are brought together.

The Findings as Disclosed by Morphological Examination of the Blood.—*The Red Blood Cells.*—The red blood cells are small discoid, possibly cup-shaped bodies. The diameter of the normal adult cell averages about 7μ, though it may vary from 6 to 9μ. Cells of normal characteristics are referred to as normocytes. In infancy a considerably greater variance is seen, with a large number of much smaller cells. By *microcytes* we mean small cells less than 6μ in diameter, and by *macrocytes* over 9μ in diameter, the term *megalocyte* ordinarily being reserved for a cell over 16μ in diameter. Detection of unusual cells is of importance in the diagnosis of the anemias. The normal red cell contains no nucleus. *Anisocytosis* is a term used to describe marked irregularity in size. In the fresh preparations the cells have a slight greenish yellow tint. After fixation, they manifest an affinity for acid stains (acidophilic, oxyphilic), and therefore when treated with stains of the Romanowsky group take a pinkish copper color, due to the action of eosin (they are eosinophilic). Normally the stained cells show practically uniform depth of color since the hemoglobin content is approximately the same.

Polychromatophilia (polychromasia) is seen in some abnormal cells. Because of degeneration or immaturity, the eosin has not been taken up well and the basic stain has been partially absorbed. Cells so stained are seen in pernicious anemias, occasionally in the leukemias, after certain poisons, in malaria and in certain other conditions. Such cells were viewed, especially by Ehrlich, as degenerated cells, but present-day opinion is inclined to regard them as immature forms.

"Cabot's ring bodies" are faint ring-like structures seen with the red cells, staining a reddish or bluish tint with the Wright's stain. Cabot considered them to be nuclear remnants. Their presence in the circulating blood might be construed as evidence of the escape into the circulation of only partially matured cells.

Howell's Nuclear Particles.—These are bits of nuclear material, too large to be called granules, having the shape and appearance of a nucleolus, usually lying at the periphery of the cell. Howell's explanation is that they are bits of the nucleus left adhering to the corpuscle at the time the nucleus escaped. The particles have been seen in experimental anemia in rabbits poisoned by pyrodin, the blood of cats following severe hemorrhage, and in pernicious anemia in man. The bodies are looked upon as signs of regeneration of the blood. They are also referred to as Howell-Jolly bodies.

Punctate basophilia (punctate basophilia of Grawitz) refers to a condition occasionally noted in red blood cells where granules of varying size and numbers may be seen, stained with the basic blue stain. The granules may be exceedingly small, so small as to be scarcely visible, while on the other hand, the size may be considerable. They are regarded by some as nuclear remnants, though this is contested by Simon, since they do not stain with methyl green, a specific nuclear dye. At any rate their occurence, with extremely rare exceptions, *is evidence of severe anemia.* They are seen in pernicious anemia, lead poisoning and malaria, occasionally in the leukemias and cachexias. Simon states that they are absent in chlorosis and the anemia of chronic nephritis. Their detection in suspected cases of lead poisoning is of prime diagnostic import. Basophilic stippling may be demonstrated with Wright's stain. The films should be stained immediately.

Shape.—The outline of the red cells as seen in stained preparations is circular or practically so. Marked deformity of the cell outline is spoken of as poikilocytosis. This is seen best in pernicious anemia. There may be variety of irregularities, so that we may see cells of oval, pear, tennis-racket, or dumb-bell shape. Before deciding that poikilocytosis be present, assurance should be had that the irregularity of cellular contour be not a mere artefact due to faults in the preparation of the film. With artefacts, the long axes of the elongated red cells are usually parallel. When poikilocytosis is actually present, it is customary to find normal cells in the same microscopic fields, while when mechanical injury is responsible entire fields of bizarre deformed cells will be found.

Nucleated Red Blood Cells.—In normal adult circulating blood nucleated red cells are never seen. They are found in fetal blood and as would be expected, are occasionally encountered in the blood of normal infants during the first few days of life. Since they are in the process of development, they are found in great numbers in the bone-marrow.

In the blood of adults with certain diseases we see various types of nucleated red cells. The *normoblast* is a cell corresponding in size to the normocyte, the diameter ranging from 6 to 9μ with a nucleus occupying a more or less central position while the protoplasm frequently shows a tendency to polychromatophilia, (Plate I, *a*) so that the color ranges from red overcast with blue to a greenish-blue. The nucleus is usually densely stained with the blue nuclear stain and little chromatin network is to be distinguished (pycnotic nucleus), though occasionally a radial arrangement of the chromatin may be made out. Mitotic figures are occasionally seen (karyokinesis).

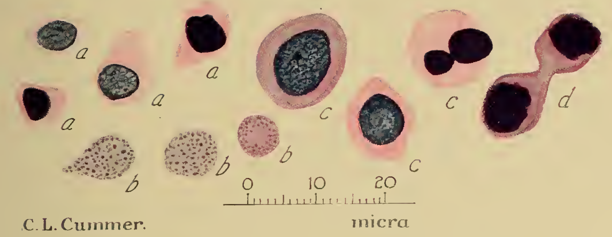
Normoblasts appear in the circulation as a result of hyperactivity on the part of the bone-marrow. They may be seen in any of the anemias when the marrow is attempting to replace blood rapidly and so turns out immature forms, or in diseases of the bone-marrow, such as splenomyelogenous leukemia, where all sorts of immature red and white cells are discharged into the circulation. When constantly absent from the bone-marrow in cases whose blood picture is otherwise typical of pernicious anemia, this fact in itself points to lack of any endeavor on the part of the bone-marrow to regenerate blood so that complex corresponds to an aplastic anemia, the prognosis of which is distinctly poor even as regards a favorable remission.

Megaloblasts.—These are nucleated red cells whose diameter ranges from 10 to 12μ up to two or three times that of a normoblast, but whose nucleus is usually of quite different character, in that it is large, is usually eccentric, and ordinarily stains more faintly, having a vesicular appearance quite in contrast to the pycnotic type common in normoblasts (Plate I, *b*). The nucleus may show many irregularities including vacuolation and subdivisions into small basophilic staining fragments. The cell outline is frequently irregular, sometimes almost polygonal, even appearing indented in ragged fashion. With cells of this type mitotic division of the nucleus is noted at times (Plate I, *d*) and here also we encounter polychromatophilic cytoplasm and punctate basophilia. The worker must be on the alert not to dismiss these cells as large mononuclear leukocytes, particularly as "Türck's irritation forms," which will be described later.

Megaloblasts escape into the circulating blood comparatively rarely and are interpreted as signs of an exceedingly grave anemia and of an arrest in the natural development of red cells. Broadly speaking they are seen in adults only in pernicious anemia or severe anemias of this type and in the leukemias; though rare cases of secondary anemia or even chlorosis are reported in which they have been seen. In the blood of young children their presence does not bring the same serious message as with adults, since the blood-forming organs in childhood responds with greater changes to much lesser stimuli.

Microblasts are extremely small red cells, the diameter being not over 3.5μ, with nuclei similar to those of normoblasts.

PLATE I



Types of Abnormal Red Blood Cells.

All cells drawn to same scale as Plates II and III for purpose of comparison. Each division of the scale at the bottom of the plate represents one micron. Magnification, 1150.

Stained with Wright's stain, *a*, normoblasts; *b*, erythrocytes showing punctate basophilia; *c*, megaloblasts; *d*, megaloblast with nucleus undergoing division.

Platelets.—In stained specimens the platelets appear as tiny oval or circular bodies about 3μ in diameter, the main substance having tinted blue surrounded by tiny reddish granules. They are often in little clusters or clumps, entangled with threads of fibrin (Plate II, *14*). While their origin is not definitely settled, general acceptance is given to Wright's explanation, that they are pinched off from the megakaryocytes, or bone-marrow giant cells. Their function is in connection with the coagulation of the blood, evidently the furnishing of prothrombin. The period of their existence is extremely short, about three days. From a practical point of view, it is extremely important to learn to recognize these little bodies, since they frequently lie over a red cell and are mistaken all too often for nuclei or malarial parasites.

Description of Leukocytes Found in Normal Blood.—*The Polymorphonuclear Neutrophilic Leukocytes.*—Commonly spoken of as "polys.," (abbreviated by many as P.M.N.) these cells are the most numerous in the circulating blood, being encountered 6 or 7 times out of every 10 leukocytes which are seen. As observed in stained specimens, they are approximately circular, though outline is oval at times. In fresh specimens, ameboid movement may be seen. The diameter is about 9 to 12μ, that is, from about one and a half times up to almost twice that of the average red cell. The nucleus is quite irregular in form, hence the name. It may be U-shaped, S-shaped, multilobular, or apparently broken into several fragments which, while apparently distinct, are in reality joined by a fine thread of nuclear material (Plate I, 1–5 incl.). Arneth has classified cells according to the divisions of the nucleus (see page 89). The nucleus always takes a basic stain, that is, it stains a blue which is not as deep a shade as that of the lymphocyte nucleus. Nucleoli are not seen.

The protoplasm is comparatively abundant. The ground substance is somewhat acidophilic, tinted pink. This is not always easy to discern since the protoplasm is studded with many fine granules which are neutrophilic and as a result stain a lilac color or not infrequently a reddish tint due, so Barker states, to the acidophilic property of the granules in older cells. Inclusion bodies within the cytoplasm of the cells have been described by Döhle. It is to be remembered that the description of the staining properties is based upon the results of Wright's stain.

The polymorphonuclear neutrophiles are the so-called "pus cells," though when seen in pus they have frequently undergone degeneration. Their function is to attack and engulf bacteria and to produce a proteolytic ferment. This ferment acts only in alkaline medium and its action is inhibited by blood serum, so that in the presence of blood serum, its proteolytic activity is carried on only within the cell. It is thought that they participate in the formation of complement.

The Polymorphonuclear Eosinophilic Leukocytes.—The cells are commonly referred to as "eosinophiles." The name is abbreviated to

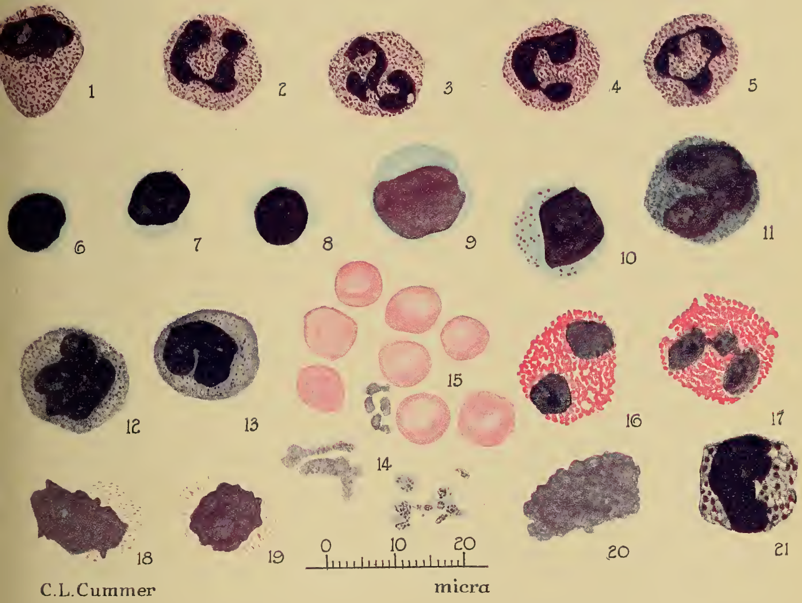
P.M.E. or E. They are cells similar in size and shape to the neutrophiles just described, though possibly slightly larger. Their number is much less, one ordinarily encountering only from 2 to 4 in counting 100 leukocytes, 5 being the upper limit of normal. The nucleus is more regular, less broken up, and stains less intensely than that of a neutrophile, frequently so feebly that it is difficult to make out. There are no nucleoli. (Pate II, *16*, *17*.) The protoplasm cannot be seen usually on account of large and uniform granules staining a bright intense red whose size and refractility renders them easily distinguishable even in unstained specimens of fresh blood. It is important to become familiar with the comparatively great size of these granules. When once mentally photographed, they should never be confounded with neutrophilic granules even if the latter have been stained a red tint rather than lilac. Barker's demonstration that the granules contain iron is of distinct interest.

Polymorphonuclear Basophilic Leukocytes.—Commonly these cells are referred to as mast-cells. The abbreviations P.M.B. or B. may be used. They are comparatively infrequent in normal blood, running usually about 0.5 per cent. In size they are smaller than the other polymorphonuclear cells, the average diameter being about 8 to 10μ. The nucleus, which occupies about two-thirds of the cell, may be quite difficult to differentiate since it stains poorly and is frequently eclipsed by the deep staining granules which may overlie it. The polymorphism of the nucleus is different from that of the two forms of cells just described, for it often has rather a knobby, knurled appearance, so that it has been described as representing the outline of a clover leaf or rosette.

The granules are of large size, indeed are practically of the same as the granules of the eosinophiles. They take the basic blue stain very intensely, manifesting an almost purple color. The protoplasm is not nearly so thickly studded with granules as with the eosinophiles, the granules are irregular in size, and the protoplasm between them appears almost colorless, occasionally faintly red or a delicate blue. (Plate II, *21*.)

The Lymphocytes.—For convenience, the abbreviation L. will be used. The proportion in normal adult blood ranges from 20 to 25 per cent. The size of these cells runs from 7 to 10μ. They contain a proportionately large nucleus, practically filling the cell, so that only a narrow rim of cytoplasm may be seen about it. This is a variable feature, however, and at times a relatively larger amount of cell substance may be present. Ordinarily the nucleus has a decided affinity for the basic stain so that its color is deep blue, staining much more deeply than the nuclei of any of the other forms of leukocytes. Occasionally a framework may be made out within the nucleus. In each nucleus one or two nucleoli are present, small, oval bodies staining blue. The protoplasm is not granular. It is feebly basophilic and in satisfactory stains is transparent and of faint sky-blue tint. At times the

PLATE II



Types of Cells Found in Normal Blood.

All cells drawn with the same magnification and outlined with the camera lucida for the purpose of comparing sizes. Each division of the scale at the bottom of the plate represents one micron. Magnification, 1150.

Stained with Wright's stain. Nos. 1 to 5, inclusive, polymorphonuclear neutrophilic leukocytes; 6, 7 and 8, small mononuclear leukocytes; 9, 10, large mononuclear leukocytes; 11, 12, 13, transitional leukocytes; 14, a group of platelets; 15, a group of red blood cells; 16, 17, polymorphonuclear eosinophilic leukocytes; 18, 19, 20, "basket cells," degenerated leukocytes; 21, "mast-cell," basophilic leukocyte.

color will be a little darker at the cell border than close to the nucleus (Plate II, *6*, *7* and *8*). With Wright's stain a few "azure granules" are seen, granules of variable size and staining red. This description applies to the small lymphocytes or the small mononuclears.

Under the heading of lymphocytes we must also include the large lymphocytes, which are identical in all respects except for greater size, the proportion between cytoplasm and nucleus being the same. These cells are relatively, sometimes absolutely, increased in typhoid fever and other infections. Apparently they are of distinct value in combating these infections, since it has been shown by Murphy and Ellis' work that animals deprived of lymphocytes by *x*-ray treatment are more susceptible to tuberculosis. On these grounds, it is assumed that the round-cell (lymphocyte) infiltration seen as a late stage of an inflammatory reaction is an index of the power of the lymphocytes to ward off injuries. The work of Webb and Williams is interesting, since they have shown that normal adults and children as well as guinea-pigs and rabbits living at high altitudes (Colorado Springs) show a high mononuclear count as compared with counts made at sea-level.

The Large Mononuclear Leukocytes.—These are dubbed "large monos." The abbreviation L.M. will be used. This form varies in size from 10 or 12μ up to 20μ. About 1 to 5 per cent. of all the leukocytes are large mononuclears. The protoplasm is conspicuously abundant, in comparison with that of the lymphocytes, occupying a distinct area about the nucleus, which is usually eccentrically placed, again in contradistinction to the centrally placed nucleus of the lymphocyte. While with ordinary staining the protoplasm has practically the same staining properties as has that of the lymphocytes, appearing as sky-blue and almost transparent, it is uniform throughout, and shows no deepening at the cell boundary. "Azure granules" are frequently seen. Again by way of contrast, the nucleus does not stain nearly as deeply as the lymphocyte nucleus. (Plate II, *9* and *10*.) At least a large proportion of these cells are phagocytic and contain a proteolytic enzyme which acts only in acid medium. They probably exert a protective function in tuberculous processes.

The Transitional Leukocytes.—The transitionals to which we will give the abbreviation T. are of large size, exceeding all the other forms in this respect. They compose about 2 to 3 per cent. of the total leukocytes. The characteristic features are their size and more particularly the presence of an indented or notched nucleus. The nuclei stain a moderately faint blue about as deep as the nucleus of the large mononuclears. The cytoplasm is faintly basophilic taking a light blue color, and contains a varying number of lilac-colored neutrophilic granules, similar in appearance to the granules of the polymorphonuclear neutrophiles and often most marked about the notch. (Plate II, *11*, *12* and *13*.) It is now felt that they are a mature form of large mononuclears, and should be grouped with the latter in finally interpreting a differential count. Their name is a misnomer since at present they are not considered as transitional forms between large mononuclears and polymorphonuclear neutrophiles.

Forms of Leukocytes Found in the Blood in Diseased States.—*The Myeloblasts.*—These are occasionally called non-granular myelocytes. In development they are the precursors of the granular myelocytes, which are in turn the forerunners of the granulated polymorphonuclear cells of normal circulating blood. The size is variable. Small forms are occasionally seen, but ordinarily the diameter is from 12 to 20μ. The nucleus is sometimes round, more frequently oval, stains a light blue, and contains a number of nucleoli. The practically clear protoplasm stains very light blue. These cells are found in the bone-marrow and may escape into the circulation only in certain pathological conditions. (Plate III, *4*, *5* and *6*.)

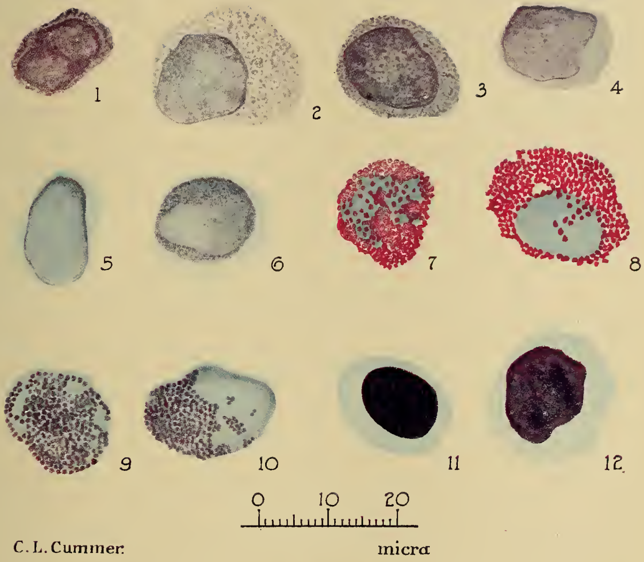
The Myelocytes.—The myelocytes are normally present in the bone-marrow and are found in the circulating blood only in pathological conditions. They are large in size, the diameter ranging from 15 to 20μ or more. The nucleus stains a rather faint blue, and contains nucleoli. Usually it is not in the center of the cell. The surrounding cell substance is conspicuous by its large amount, is slightly basophilic (blue) and studded with granules, which may be eosinophilic (staining red), basophilic (staining blue) or neutrophilic (faint reddish-blue or lilac staining). Accordingly the myelocytes are classified respectively as eosinophilic myelocytes, basophilic myelocytes, or neutrophilic myelocytes. With the first two forms, the granules are large, while the granules of the latter are small, corresponding in size to the granules of the similar granulated polymorphonuclear cells of normal blood which are descended from them. They arise from myeloblasts of the bone-marrow. An interesting variant is an occasional cell containing both eosinophilic and basophilic granules. (Barker attributes this to the youth of the granules.) Usually in leukemic bloods the neutrophilic myelocytes predominate. While the myelocytes often are divided into these classes in executing a differential count, it must be admitted that no especially useful purpose is served by so doing. (Plate III, *1*, *2*, *3*, *7*, *8*, *9*, *10*.)

We must remember that all of these cells are merely stages in the process of development of the polymorphonuclear cell of normal blood from the myeloblasts and that many transitional forms will be encountered in leukemic bloods.

Türck's "Irritation Forms."—This is a large mononuclear cell resembling the myeloblasts in size, shape, and in the character of the nucleus. The protoplasm, however, stains a deep reddish-blue and is not infrequently vacuolated. It is considered by some to be a pathological myeloblast and is seen in the circulation in severe infections, in severe anemias and in myelogenous leukemia.

The Megakaryocytes or Bone-marrow Giant Cells.—These cells seldom escape into the circulation. They are of great size and have large and convoluted nuclei. Occasionally multiple nuclei are seen.

PLATE III



C. L. Cummer.

Leukocytes Found in Circulating Blood Under Abnormal Conditions. (Stained with Wright's Stain.)

All cells drawn with camera lucida with the same magnification and to the same scale as Plates I and II for the purpose of comparison. Each division of the scale at the bottom of the plate represents one micron. Magnification, 1150.

Nos. 1, 2 and 3, neutrophilic myelocytes; 4, 5 and 6, immature myelocytes, non-granular, with basophilic myelocytes; 11 and 12, lymphoblasts.

Cells of Lymphadenoid Origin.—The cells just described (myeloblasts, myelocytes, Türck's "irritation forms" and megakaryocytes) are of myeloid of bone-marrow origin. Under other abnormal circumstances we may see cells discharged into the circulation which have come from the lymphatic tissue.

Ordinary Lymphoblasts.—These are essentially similar to the ordinary lymphocytes, though somewhat larger in size and possessed of more protoplasm. They have their origin in the germinal centers. They may be seen in the blood of normal children but in adults' blood only under pathological conditions. (Plate III, *11* and *12*.)

Pathological Lymphoblasts (*Rieder's Cells*).—These are lymphoblasts of large size, seen occasionally in acute lymphatic leukemia, at times in the chronic form, in various infectious diseases and Basedow's disease. The nucleus is large, and there is little protoplasm, the nucleus often coming to the edge of the cell on one side. "Azure granules" may be seen in the cytoplasm. (Plate III, *11* and *12*.)

The Formation of Blood.—*Introductory.*—Before entering into a consideration of the anemias and leukemias, it is necessary to review the formation of the blood cells and their life-history. In the embryo the primitive cells apparently are formed from the endothelial cells of the capillaries. Later the majority of the cells in the fetal circulation are hemoglobin-bearers, while the leukocytes are present in small numbers. Practically all the hemoglobin-bearing cells contain nuclei and are produced in various organs, particularly the liver. About the third month of fetal life, the formation of bone-marrow begins and this gradually supplants all other organs in the production of all cells except those of lymphocytic origin. The bone-marrow produces both large and small erythroblasts and later non-nucleated red blood cells. The polymorphonuclear cells appear comparatively late. At the time of birth, the blood contains non-nucleated red cells, neutrophilic leukocytes, lymphocytes, and a few normoblasts, neutrophilic myelocytes and myeloblasts. Eosinophilic and basophilic leukocytes are rare. Soon after birth the normoblasts, myeloblasts and myelocytes disappear from the circulation to reappear only under abnormal conditions.

In the adult we have under normal conditions as blood-forming (hematopoietic) organs probably only the bone-marrow and the lymphoid tissue. It is possible that under abnormal conditions, when the bone-marrow is unable to produce the red cells in sufficient numbers, a certain amount of red cell formation occurs in the spleen. In myelogenous leukemia the liver, spleen and other organs may contain deposits of myelocytes, which are presumed to be production centers for these cells.[1]

The Bone-marrow.—An understanding of the formation of the blood is greatly facilitated by a study of the bone-marrow. As was stated, this does not begin to function until the third or fourth month of fetal life, after which time it is quite active. At birth the shafts of the long bones are filled with red marrow, but during the period of childhood this is gradually replaced by fatty tissue, leaving only small deposits of actual marrow in the diaphyses of the long bones, in the bodies of the vertebra and the cartilaginous ends of the ribs. When severe anemia occurs and there is a need for replacement of blood or in myeloid leukemia, the fatty marrow may be displaced by actively proliferating marrow, containing red and white cells and their precursors.

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[1] For a discussion of the pathological physiology of blood-cell formation and blood-cell destruction, the student is advised to consult the article by Cecil K. Drinker in The Oxford Medicine, Oxford University Press, New York and London, 1920, ii, 509.

For the purpose of study, a portion of the bone from the middle of the shaft of the tibia should be obtained at autopsy, as soon as possible after death. It should be cut into with bone forceps and a little of the marrow squeezed out on a cover-slip, mixed with a droplet of physiological salt solution and carefully spread as directed for the preparation of blood smears with cover-slip method. The resulting film should be stained with Wright's stain and carefully studied. In it will be found all the cells which are found in normal circulating blood, since it is needless to say that this has access to the bone-marrow, and in addition, the cells found in the marrow only. Therefore the following should be seen:

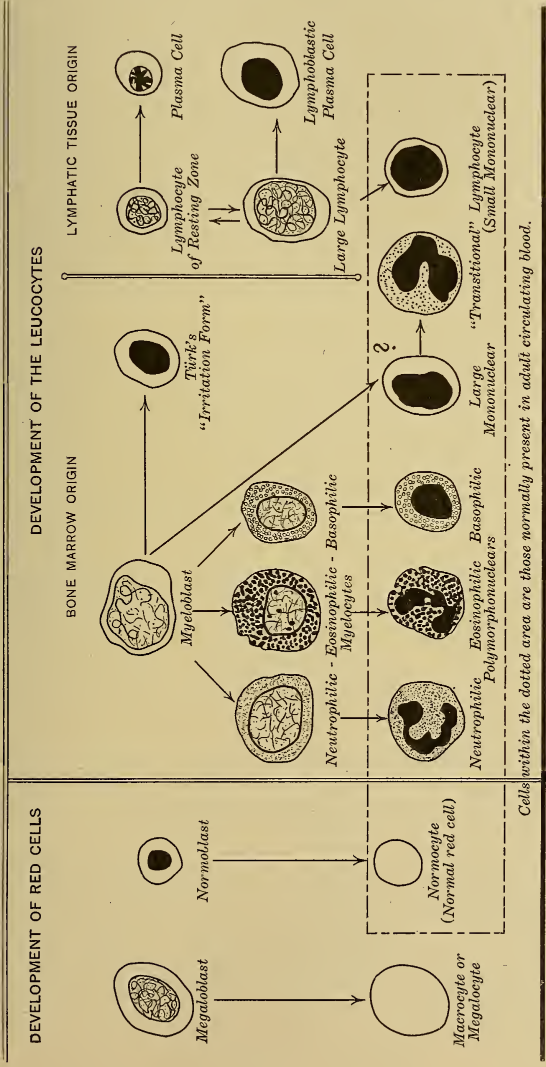
1. Red cells in all stages of development, normoblasts with one or more nuclei, an occasional megaloblast, and the normal red cells which we see in circulating blood.

2. White cells as seen in circulating blood, polymorphonuclear cells with neutrophilic, eosinophilic, and basophilic granules, lymphocytes, large mononuclears, and transitional cells.

3. White cells which are seen in the circulation only in disease but are normally present in bone-marrow, giant marrow-cells (megakaryocytes), myeloblasts, myelocytes with neutrophilic, basophilic and eosinophilic granules and other border-line forms which may be difficult to classify.

The Origin of the Different Forms of Cell.—The polymorphonuclear cells, regardless of the type of granulation, have their origin in the bone-marrow and are derived from neutrophilic myelocytes which in turn are derived from the non-granular myeloblasts. As has been stated all these forms, and hence all stages of development, may be seen in preparations from bone-marrow. The genealogy of a polymorphonuclear neutrophilic leukocyte is myeloblast, neutrophilic myelocyte, neutrophilic polymorphonuclear leukocyte (Fig. 21).

Many *large mononuclear cells* likewise have their origin in myeloid tissue, and *transitional cells* are now presumed to be a later stage. Some of the large mononuclear cells probably arise from the endothelial cells of the capillaries, since oxydase granules cannot be demonstrated in all of these cells. While there can be little doubt that a certain proportion of the large mononuclear cells come from the endothelial cells of the capillaries, it is equally certain that certain of the large mononuclears are of myeloid origin because they contain oxydase granules. Therefore it is not fitting to apply the term endothelial leukocytes to all the large mononuclears, but rather to retain the generally accepted terminology here given, remembering that it includes two types of cells having similar appearance but different sources. The transitionals are not, as their misleading name would



Cells within the dotted area are those normally present in adult circulating blood.

* *Adapted from Barker, "Monographic Medicine", Vol. III, modified from O. Naegeli.*

Fig. 21

imply, a transitional stage previous to development into polymorphonuclear cells.

The lymphocytes are of lymphadenoid origin, arising from lymph-glands, splenic Malpighian follicles, and lymph nodules.

Life History of the Red Blood Cells.—It is of interest to follow the history of the red cells. Their duration of life was estimated by Quincke as one month. The work of Ashby, tracing the life of transfused blood in the circulation of the recipient by means of agglutination, shows that their life is for thirty days or more. The birthplace is the bone-marrow and their "graveyard" is chiefly the spleen, where damaged or dying cells are caught by the macrophages. This function is performed to a lesser extent by bone-marrow and liver and is taken over by the lymph nodes after splenectomy, as has been shown by Pearce. After destruction the non-iron bearing pigment is carried to the liver, where it is transformed to biliary pigments and is so excreted via the bile ducts and intestinal tract.

Life History of the White Cells.—The tenure of life of a leukocyte is unknown. Many of course are lost in combating infection and by way of various secretions (mouth, nose, throat, etc.). In leukemia the destruction of leukocytes is tremendous and because of the breaking down of nucleoproteins, the endogenous uric acid is greatly increased, as is nitrogen-metabolism in general.

TABLE SHOWING FORMS OF ERYTHROCYTES, LEUKOCYTES, PLACES OF ORIGIN, AND CONDITIONS OF PRESENCE IN CIRCULATING BLOOD.

Form of cells.	Origin.	Present in bone-marrow?	Present in normal circulating blood?	Ever present in circulating blood in pathological condition?
Erythrocytes.				
Normocyte	Bone-marrow	Yes	Yes	Yes
Normoblast	Bone-marrow	Yes	No	Yes
Megaloblast	Bone-marrow	Yes	No	Yes
Leukocytes.				
Polymorphonuclear neutrophiles	Bone-marrow	Yes	Yes	Yes
Polymorphonuclear eosinophiles	Bone-marrow	Yes	Yes	Yes
Polymorphonuclear basophiles	Bone-marrow	Yes	Yes	Yes
Mononuclears, large . . .	Bone-marrow	Yes	Yes	Yes
"Transitional" cells . . .	Bone-marrow	Yes	Yes	Yes
Mononuclears, small . . .	Lymphoid tissue	Yes	Yes	Yes
Ordinary lymphoblasts . .	Lymphoid tissue	Yes	No[1]	Yes
Pathological lymphoblasts (Rieder's cells)	Lymphoid tissue	Yes?	No	Yes
Megakarocytes	Bone-marrow	Yes	No	Seldom
Myeloblasts	Bone-marrow	Yes	No	Yes
Türck's "irritation-cells" . .	Bone-marrow	Yes	No	Yes
Myelocytes, basophilic . . .	Bone-marrow	Yes	No	Yes
Myelocytes, neutrophilic . .	Bone-marrow	Yes	No	Yes
Myelocytes, eosinophilic . .	Bone-marrow	Yes	No	Yes

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[1] Occasionally, in childhood only.

Theories in Regard to Blood Formation.—It has been noted that we have assumed a double origin for the blood cells, the formation of a portion in the bone marrow and a portion in the lymphadenoid tissue, that in each instance all cells formed in a given tissue have had a common ancestor, but that these two common ancestor-cells were quite distinct. It should be stated that while this theory of Ehrlich's has been adopted by many hematologists it is not universally supported and that there is a considerable school which follows the lead of Pappenheim in regarding all leukocytes as descended from a common ancestor cell. In our opinion very strong evidence in support of the theory of Ehrlich is the demonstration of oxydase granules in the cells of bone-marrow origin and their absence in the lymphocytes. Furthermore it would seem that the theory of Pappenheim, going a step further than Ehrlich's, requires much additional proof which has not been forthcoming in convincing form.

THE LIFE HISTORY OF THE RED BLOOD CELLS.

Place of origin.	Duration of life.	Function.	Place of destruction.	Disposal of end-products.
Red blood cells	One month or more	Oxygen-bearing	Spleen chiefly; also liver and bone-marrow	Non-iron-bearing pigment carried to liver, here transformed to biliary pigments, excreted as bile via intestinal tract.
Leukocytes	Unknown	Combating infection; proteolytic action, etc.	In blood stream	Nitrogen from nucleo-protein excreted by urine.
			In exudates	Lost in secretions from mouth, nose, throat, etc.

THE DIFFERENTIAL COUNT.

The directions for making a differential count of the leukocytes have been given in a preceding section. For study of the data so secured, the all-important requisite is a normal standard. The standards long given have impressed many as not at all approximating the average as obtained in counts on apparently normal subjects.

The wide variation in standards set down by different workers is well illustrated by the table given on page 64.

Form.	Ehrlich and Lazarus.[1]	Stengel.	Cabot.	Mallory.	Wood.	Simon.	Emerson.	Webster.	Bunting.[8]	Miller.	Todd.	Sondern.[9]
P. M. N. Per cent.	70–72	60–74[2]	62–70	70–72	65–75	60–70	70–72	65–75	50–60	64.2	60–70	62.0
P. M. E. Per cent.	2–4	1–5	0.5–4	2–4	0.6–11	1–4	2–4	2–4	0.8–4.0	2.8	1–4	1.0
P. M. B. Per cent.	0.5	1	1/40–1/90	0.5	0.5	0.5–1	0.5	0.5	0.4–4.0	0.6	0.5–1	0.2
L. Per cent.	22–25	25	20–30	22–25	22–25	20–30	22–25	20–25	30–40	22.4	25–33	28.0
L. M. Per cent.	1	5–8	4–8[5]	2–4[6]	1	1–6	1	3–5	0–6–2.0	8.0	2–5	7.5
T. Per cent.	1–3	[4]	[4]	[7]	2–4	[4]	1.3	[4]	6.0–8.0	2.0	[4]	

It would seem that the most extensive work in this country has been done by Miller, who has performed 650 counts on 230 normal persons. The figures which he takes as representing an average are given in the table above, and by Sondern, who performed 500 counts. Combining their figures, we should suggest the following as a normal standard:

Polymorphonuclear neutrophiles	62.0 to 64.0	per cent.
Lymphocytes	22.0 to 28.0	"
Large mononuclears and transitionals	7.5 to 10.0	"
Polymorphonuclear eosinophiles	1.0 to 3.0	"
Polymorphonuclear basophiles (mast-cells)	0.2 to 0.6	"

The Leukocytoses.—By leukocytosis (*hyperleukocytosis*) is meant a transitory increase in the number of leukocytes in the circulating blood above the normal maximum figure. For example, 12,000 white cells per cmm. would be a leukocytosis. By *absolute leukocytosis* is meant that the actual number of leukocytes per cmm. is greater than normal, while by the *relative leukocytosis* is meant that a certain type is relatively increased with respect to the differential count without a marked change in the total count. *Leukopenia* refers to a scarcity in the white blood cells such that the total number is below the normal minimum. A count of 3500 or 4000 would represent a leukopenia. This occasionally is referred to as hypoleukocytosis.

The leukocytoses may be divided according to form of cell which is increased. A leukocytosis is ordinarily accompanied by disturbance in the proportions of the differential count, so that there is a greater percentage than customary of one or more forms of cell. Accordingly we have a polymorphonuclear neutrophilic leukocytosis, an eosinophilic leukocytosis (eosinophilia), a lymphocytosis, etc.

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[1] Nothnagel's Encylopedia of Practical Medicine, Am. ed.

[2] States that there are 65 to 75 per cent. polymorpnonuclear forms (neutrophiles and eosinophiles) and that 1 to 3 or 5 per cent. of the leukocytes contain eosinophilic granules, and that occasionally a larger number is met with in normal blood.

[3] Not given.

[4] Included with large mononuclears.

[5] Termed by Cabot "large lymphocytes" and including transitional cells.

[6] Termed "endothelial leukocytes."

[7] Not stated.

[8] Am. Jour. Med. Sc., cxlii, 699–702.

[9] Medical Record, March 25, 1905, lxvii, 453.

It may well be that Bunting's figures are due to the fact that he was dealing with inhabitants of the Great Lakes District, where chronic catarrhal conditions are extremely prevalent.

The influence of altitude evidently must be considered since Webb and Williams have shown that percentage of mononuclears is higher in those living at high altitudes (Tr. Nat. Assn. for Study and Prevent. of Tuberculosis, 1909).

Physiologically, we may have a polymorphonuclear neutrophilic leukocytosis in pregnancy, after a cold bath, during digestion, and after exercise. It is not likely that the count will be increased over 12,000 or 14,000. It should be determined whether any of the factors are present, and if so, due allowances should be made.

Pathological Leukocytosis.—These are of distinct importance in differential diagnosis. The essentials may be presented in the following summary.

Polymorphonuclear eosinophilic leukocytosis is found in infection with certain intestinal parasites, *e. g.*, Anchylostoma duodenale, Oxyuris vermicularis, Ascaris lumbricoides, and the tenias, in generalized round-worm invasions, such as trichinosis and filariasis, and also in echinococcus cyst. It is observed in scarlet fever and is seen occasionally in malignant tumors, especially bone-marrow tumors. It is expected in postfebrile conditions where there has been a decided neutrophilic leukocytosis. Eosinophilia is found after the use of certain drugs, such as camphor, and after injections of tuberculin. It is found in bronchial asthma, in certain skin diseases such as herpes zoster, pemphigus, extensive psoriasis, prurigo, dermatitis herpetiformis, gonococcic invasion of posterior urethra and epididymis, and myelogenous leukemia.

Polymorphonuclear neutrophilic leukocytosis is found in the majority of acute infections, such as small-pox, scarlet fever, acute articular rheumatism, meningitis (including tuberculous meningitis), cholera, erysipelas, diphtheria. In general, a polymorphonuclear leukocytosis occurs in infections due to the streptococci, the staphylococci, the meningococci, colon bacilli and pneumococci. It is seen after hemorrhage, particularly after post-operative hemorrhage, where there is usually a rapid drop to normal, in eclampsia, and after the use of certain drugs, such as quinine, salicylates, saline infusion, ether and chloroform inhalations, and potassium chlorate in large doses. It is occasionally noted with certain malignant growths. In general, neutrophilic leukocytosis is seen in abscess and suppurative conditions, unless the process is well walled-off. In myelogenous leukemia, the actual number of polymorphonuclear neutrophiles is greatly increased, though the percentage may be low, on account of the overwhelming number of myelocytes.

Basophilic leukocytosis is seen practically only in myelogenous leukemia.

Lymphocytosis.—An absolute lymphocytosis is seen in lymphatic leukemia and often in pertussis, where the count may reach 20,000. In infancy and childhood it should be remembered the normal lymphocyte count is relatively high. A relative lymphocytosis is seen in pertussis (absolute lymphocytosis also), chronic infectious diseases, exophthalmic goiter, pernicious anemia, splenic anemia, cirrhosis of the liver, rickets, typhoid fever, measles, uncomplicated tuberculosis, influenza, and congenital syphilis.

Large mononuclear leukocytosis is not frequently seen, though it is occasionally noted in severe cases of malaria, the cachexia of carcinoma, Hodgkin's disease, and lymphosarcoma.

VARIATIONS IN DIFFERENTIAL COUNT OF LEUKOCYTES.

NEUTROPHILIC LEUKOCYTOSIS.
(Normally, 60 to 64 per cent.)

Cholera.
Small-pox.
Infection by:
  (*a*) Streptococci.
  (*b*) Staphylococci.
  (*c*) Meningococci.
  (*d*) Colon bacilli.
  (*e*) Pneumococci.
Erysipelas.
Diphtheria.
Meningitis, including t.b. meningitis.
Scarlet fever.
Tuberculosis with marked sepsis.
Abscesses, including empyema.
Majority of purulent wound infections.
Eclampsia.
After hemorrhages.
Following use of certain drugs (inhalations of chloroform, ether and use of quinin, salicylates, phenacetin, etc.

A neutrophilic leukopenia ordinarily is found when a small mononuclear leukocytosis is present.

SMALL MONONUCLEAR LEUKOCYTOSIS (LYMPHOCYTOSIS).
(Normally, 20 to 25 per cent.)

Absolute increase:
  Lymphatic leukemia.
Increase usually only relative:
  Chronic infectious diseases.
  Pernicious anemia.
  Splenic anemia.
  Diseases of the ductless glands.
  Tuberculosis, when uncomplicated.
  Whooping-cough.
  Typhoid fever.
  Influenza.
  Rickets.
  Measles.
  Congenital syphilis.
  Cirrhosis of the liver.

A small mononuclear leukopenia ordinarily is found in the conditions characterized by a neutrophilic leukocytosis.

EOSINOPHILIC LEUKOCYTOSIS (EOSINOPHILIA).
(Normally, 2 to 5 per cent.).

Increase usually relative:
  If there is an actual numerical increase, it is slight.
Certain intestinal parasites (especially hook worm; occasionally oxyuris, ascaris, and the tænias.
Trichinosis.
Gonorrhea, particularly when the posterior urethra is involved.
Certain skin diseases, notably prurigo; zoster, pemphigus, dermatitis herpetiformis.
Scarlet fever.
Bronchial asthma.
During convalescence from diseases accompanied by neutrophilic leukocytosis.
In some cases of tuberculosis, and after injection of tuberculin.
Myelogenous leukemia.

An eosinophilic leukopenia is usually found when there is a neutrophilic leukocytosis.

LARGE MONONUCLEAR LEUKOCYTOSIS.

Increase usually only relative:
  Malaria cachexia.
  Cachexia of carcinoma.
  Hodgkin's disease.
  Lymphosarcoma.

THE BLOOD IN CHILDHOOD AND INFANCY.

We will recapitulate briefly what has been said on this subject under different headings with certain additions, in order to present a fairly complete summary so that blood findings may be evaluated properly. Rigid figures cannot be given, since development is not a uniform matter and diverse factors may alter findings even in obviously normal children. The red count may be as high as 7,000,000 cells per cm. immediately after birth but is usually about 5,700,000. During the nursing period the average is about 5,580,000, this count decreasing gradually until the proximity of the tenth year, when it reaches 5,100,000, after which time a slight increase may be seen up to 5,500,000.

The hemoglobin at birth is high, about 135 per cent. according to Williamson, dropping rather rapidly to about 75 to 80 per cent. from the third to fifth months, and remaining near this figure until the sixth year when it tends to gain steadily until the adult standard is attained at the tenth year.

The total white cell count is usually in the neighborhood of 20,000 to 30,000 or more during the first few days of life. During the nursing period, the cells average about 12,000, and a general tendency toward diminution is seen. At the sixth year, the count may range from 5400 to 12,400. The child at the age of fifteen usually presents the adult findings in this particular.

The differential count is quite unlike that encountered in adults. It has been shown by Warfield that immediately after birth the polymorphonuclear neutrophiles constitute about 70 to 80 per cent. on the first day and it is not until the eleventh day when 40 per cent. or more of mononuclears are seen. The findings from the sixth to the twelfth month run about 28 to 35 per cent. for the polymorphonuclear neutrophiles and 50 to 59 per cent. for the small mononuclears, with a steady tendency to assume the adult standard so that the polymorphonuclear neutrophiles take the lead at about the third or fourth year, running in the neighborhood of 45 per cent. with the small mononuclears about 39 per cent. This tendency is maintained so that at or about the fifteenth year the adult differential may be expected. Lucas has called attention to a change in the blood picture in measles. This comes shortly after infection and precedes the earliest signs, *i. e.*, Koplik's spots, coryza, or eruption. The leukocyte count falls and there is a reversal of the usual relative proportion between neutrophiles and mononuclears so that the neutrophiles exceed the mononuclears.

The general preponderance of lymphocytes in infancy and early childhood is due to the greater activity of the lymphadenoid apparatus. Furthermore it is to be remembered that the blood in children responds to all stimuli much more readily than with adults and that consequently high leukocytoses are often seen with comparatively trivial infections.

Moreover, the blood-forming organs may discharge into the circulating blood cells (myelocytes, etc.) which are not seen in adults except under much graver circumstances.

SPECIAL METHODS OF EXAMINING BLOOD.

Microchemical Blood Reactions.—Data of much importance is secured by certain microchemical reactions. The demonstration of oxydase granules in particular is of importance since it aids us demonstrating the origin of the leukocytes.

Certain leukocytes contain oxydase granules which have the power of causing the oxidation of organic substances in the presence of an oxidizing reagent. Cells of myeloid origin will give this reaction staining blue granules while lymphocytes and lymphoblasts will not.

Graham's original method may be employed.

The Oxydase Reaction.—Solutions required:

Solution A	95 per cent. alcohol	9 parts
	Formaldehyde solution (40 per cent. gas), freshly prepared	1 part
Solution B	Alpha-naphthol (Merck's "Recryst" or Merck's "Reagent")	1 gm.
	H_2O_2	100 cc.
	40 per cent. alcohol	0.2 cc.
Solution C	Pyronin	1 gm.
	Anilin	4 cc.
	40 per cent. alcohol	96 cc.
Solution D	(0.5 per cent. aqueous solution Methylene Blue—Grubler's BX.)	

Procedure.—The films should be fixed by covering them with the fixative, Solution A. After two minutes, this is washed off with water, and the film flooded with Solution B. This is washed off and the film is allowed to remain in a dish of running water for fifteen minutes. It is then dried and stained for two minutes in Solution C. It is again washed in water and Solution D is poured on and allowed to remain from thirty to sixty seconds. After washing with water, the slide is blotted and mounted in neutral balsam.

All cells of myeloid origin except the basophilic myelocytes and mast-cells show blue granules, while lymphocytes and lymphoblasts do not.

Graham's Second Method.—On account of its more ready availability, Graham has suggested the substitution of benzidine for alpha-naphthol, dissolving a few crystals (as much as may be taken up on the tip of a small knife-blade) of benzidine in 10 cc. of 40 per cent. alcohol, adding 0.02 cc. of hydrogen peroxide. The blood smear must be fresh and should be fixed with the formaldehyde-alcohol fixative described in the previous method as Solution A. This, it is to be noted, should be freshly prepared. After it has been allowed to act for a minute or two, it is washed off under the tap and replaced with the benzidine solution, which is allowed to remain for five or ten minutes. This in turn is washed off with tap-water and followed with Loeffler's methylene blue, which is washed off after thirty seconds.

With this method the neutrophilic and eosinophilic granules stain a warm brown color. The color which appears first is greenish-blue, but this remains only momentarily. The neutrophilic granules are small and fine and tend to occur in linear aggregations. The eosinophilic granule is large and the stained substance appears to be held in a mantle or envelope surrounding a relatively unstained central substance. The basophilic granules of the mast-cells apparently do not react though rare cells, apparently mast-cells, have shown a few scattered granules of dense greenish-brown color. The large mononuclear and transitional cells often have a few indefinite granules of brown color.

Vital Staining.—*Demonstration of Skeined or Reticulated Staining.*—The demonstration of reticulated erythrocytes is the most important application of vital staining methods. The stain is readily prepared, for it is necessary only to dissolve a few grains of brilliant cresyl blue in 1 cc. of normal salt solution, adding a crystal or two of potassium or sodium oxalate to prevent rouleaux formation. If the method is utilized frequently, a 2 per cent. solution of sodium oxalate in normal salt solution may be kept on hand, but the brilliant cresyl blue must be added before use. There should be sufficient dye to have the solution just translucent in a test-tube 1 cm. in diameter.

A few drops of blood are taken from the finger or ear and allowed to flow into the tube of stain. After gently shaking the mixture is put aside for ten to fifteen minutes when a drop of the sediment is secured with a capillary pipette, placed on a slide, covered with a cover-slip, and examined with the oil-immersion lens. The reticulated cells are searched for and their proportion to normal non-reticulated cell is ascertained. In any blood there should be visible blue stained leukocytes, platelets and hemaconia, but reticulated red cells are seen very rarely (less than 1 per cent.) or not at all. They will be recognized very readily by the fact that they are somewhat larger than normal erythrocytes and show blue stained reticulation.

On account of the increasing difficulty in obtaining brilliant cresyl blue, Buckman and Hallisey have recommended substituting crystal violet (penta-methyl-para-rosanilin-hydro-chloride). This stains the platelets and the reticular substance of the erythrocytes deep violet.

Cecil Drinker recommends as the simplest method, placing a drop of 1:300 solution of cresyl blue upon a slide and allowing it to dry. A drop of fresh blood may be placed upon the slide and a cover-slip quickly adjusted and rimmed with vaseline. The dried stain is taken up quite rapidly.

Reticulated cells are slightly increased in anemia of any severity regardless of type. The increase, however, is quite overshadowed by that seen in hemolytic jaundice, when the percentages are frequently above 5 per cent. and may reach 15 to 20 per cent. (Pearce). In pernicious anemia the proportion varies from 0 to 20 per cent. (Lee, Minot, and Vincent).

The exact significance of the cells is in doubt, the decision resting between nuclear remains of immature cells or products of degeneration as part of a disease process. The weight of opinion inclines toward regarding reticulation as an evidence of cellular youth.

Staining of Mitochondria in Lymphocytes.—Mitochondria in the lymphocytes may be demonstrated *intra vitam* by drawing blood into a solution of 1:10,000 janus green (diethyl safranin-azodi-methyl-anilin). The mitochondria appear as small granules or rod-shaped structures in the cytoplasm. Cowdry states that those seen with vital staining and those demonstrated in fixed and stained preparations have a similar appearance.

Determination of Coagulation Time.—The determination of clotting time is of distinct clinical importance, especially as a pre-operative measure in jaundiced individuals or where hemophilia is suspected. Of a number of methods which may be employed, it would be immaterial which one were to be chosen if adaptability and cost of equipment were not items of consideration, provided that the worker be thoroughly informed as to the range of normal findings given by the method which he employs.

We give decided preference to the method of Lee and White, on account of its simplicity and the inexpensiveness of apparatus required, because it can be used at the bedside without a microscope, and because the blood is secured without the admixture of tissue juices.

The apparatus required is a small all-glass syringe of 15 or 20-minim (or 1 cc.) capacity with sharp needle to fit, a small glass test-tube whose bore is 8 mm., sterile physiological saline solution (0.9 per cent.), and the usual articles for preparing for venipuncture.

The syringe and needle are boiled and after cooling are rinsed by drawing in and ejecting the sterile normal salt solution. The test-tube is also rinsed out with normal salt solution. Then the skin is sterilized over the veins at the bend of the elbow, preferably with tincture of iodine followed with alcohol, and blood is obtained with the syringe by venipuncture as described elsewhere (page 126). Exactly 1 cc. of blood should be removed and ejected at once into the prepared test-tube, noting the time. The tube is now tipped slightly endwise every fifteen seconds until the blood no longer flows but maintains its surface contour when inverted. Lee and White have found that the temperature is between 65° and 90° F.

According to this method, the normal coagulation time varies from five to eight minutes, with six and a half as an average. Increasing the diameter of the tube lengthens the coagulation time. For example, Minot and Denny showed that the normal time with a tube 9 mm. in diameter is from six to eleven minutes.

If a specimen of blood be withdrawn by venipuncture into a miniature Lee and Vincent paraffined transfusion tube for enumeration of platelets, erythrocytes, and leukocytes by the Buckman and Hallisey method (see page 40), a 1 cc. portion may be withdrawn at once with a syringe and placed in a tube of 8 mm. bore to determine the clotting time.

Simple Slide Method.—This method has the advantage of extreme simplicity, but is open to the objection that variable and unknown amounts of tissue juice are added to the blood, which may materially alter the clotting time. An ordinary microscopic slide is cleaned thoroughly with soap and water and with alcohol and dried. The ear or finger tip is punctured in such manner that the blood will come freely with the least possible pressure and a series of eight drops are now placed on the slide, noting the time. At the end of one minute a clean, sharp, fine sewing-needle is drawn through the first drop, at the end of the two minutes through the second, and so on, until a drop is found which is picked up by the point of the needle due to a formation of fibrin.

Howell's Method.—A method similar to Lee and White's has been proposed by Howell, according to which 2 to 4 cc. of blood are taken and placed in a wide tube (21 mm. in diameter). Coagulation is determined by inverting as with Lee and White technic. With this quantity and a tube of this size, Howell gives the coagulation time in normal individuals as twenty to forty minutes, in hemophiliacs as from two and a half to five hours. He states that there is no difficulty after venipuncture in hemophiliac patients provided that the vein be not wounded.

Determination of the Bleeding Time.—The coagulation time does not always correspond to the bleeding time, probably because of the thromboplastin content of the tissues. Duke suggests the following method for ascertaining the bleeding time: A small cut is made in the lobe of the ear and at half-minute intervals the blood is blotted up with filter paper. Each blot represents a half minute's flow of blood, and the decrease in the size of the drops is an index to the decrease in the hemorrhage. The cut should be such that the diameter of the first blot is 1 to 2 cm. The total duration of the hemorrhage is called the bleeding time. Normally, it varies from one to three minutes.

Determination of the Amount of Prothrombin.—Howell has shown that the amount of prothrombin is deficient in hemophiliacs. The method which he has devised is as follows: The specimens of blood are obtained from the superficial veins of the arm by means of a graduated syringe of the Luer type. The syringe is of course sterilized before use and is partly filled with sterile normal (0.9 per cent. sodium chloride) solution. This is expelled so as to leave the needle full in order that the barrel may contain no air, since a bubble or two interferes with the exact reading. 4 cc. of blood are ejected from the syringe at once into a centrifuge tube containing 0.5 cc. of oxalated normal salt solution (1 per cent. of sodium oxalate made up in 0.9 per cent. sodium chloride solution). The blood is mixed with the oxalate solution by inverting the tube, and is then centrifugalized sufficiently to obtain perfectly clear plasma. The plasma is pipetted off and 5 drops are placed in each of four tubes. To these tubes are added respectively, 2, 3, 4, and 5 drops of 0.5 per cent. calcium chloride solution. The clotting time of normal plasma is quite uniform, varying between nine and twelve minutes in the tubes to which 2 or 3 drops of the calcium chloride have been added. In setting up the test, however, it is necessary to set up a parallel control with normal blood and to subject it to the same treatment throughout, since the coagulation time may be materially modified by the preliminary centrifugalization.

The clotting time for oxalated serum to which the optimum amount of calcium chloride had been added was markedly prolonged in Howell's cases of hemophilia, ranging from ninety to two hundred and forty minutes. There was no variation from normal in purpura hemorrhagica or the other purpuras. The determination of prothrombin is of value in differentiating hemophilia from other hemorrhagic conditions, and is recommended as a preliminary to operation on a patient exhibiting the hemophilic tendency.

Resistance of Red Cells to Hypotonic Salt Solution.—The method is based on the fact that though in isotonic salt solution normal red cells remain for a period of several hours without damage, normal cells are quickly dissolved in distilled water, and hemoglobin is liberated so that the fluid is tinged bright red. If intermediate strengths of salt solution are arranged, the exact dilution which is required to cause hemolysis can be ascertained. This is found to be a variable factor.

A method which has the advantage of requiring the use of only one accurately prepared solution is that proposed by Giffin and Sanford, who have modified the technic originally proposed by Ribierre.

A stock solution is made with great care, dissolving 0.5 gm. sodium chloride in 100 cc. of distilled water. A good balance and a volumetric flask are prerequisites. A Wassermann rack with two rows of holes, twelve holes in a row, is filled with tubes. The front row is used for the patient's blood, the back for the control. The tubes in the front row are marked with a series of numbers, 25, 24, 23, 22, 21, 20, 19, and so forth to 14. In each tube is placed the number of drops of 0.5 per cent. sodium chloride solution indicated by the number on the tubes, placing likewise the same number of drops in the corresponding rear control tube, holding the pipette upright in the same position throughout. For placing the salt in the tubes a capillary pipette is drawn from glass tubing or a Mohr pipette may be utilized. This same pipette is used for adding distilled water to each tube, though of course it should be carefully washed out with distilled water after using it for salt. Enough water should be added to bring the number of total drops in each tube up to 25. For example, the tube marked 20 should contain 20 drops of the saline solution and 5 drops of distilled water.

The patient is bled by puncturing the vein in the manner described elsewhere (page 126). An all-glass syringe is to be employed and it should be free from moisture. One drop of the patient's blood is allowed to flow from the syringe directly into each of the front tubes and one drop of the control blood, secured from a normal individual, into each of the rear tubes. The tubes should be shaken immediately after the addition of the blood. It is highly important that the needles used for obtaining the unknown blood and the control blood be of the same bore, when the blood is dropped into the tubes of salt solution directly from the syringes.

While the blood may be dropped directly from the syringe into the test-tubes, a better method is to secure a definite quantity (about 2 or 3 cc.) by venipuncture with a graduated syringe and to place it at once in a 15 cc. graduated centrifuge tube containing 5 cc. of citrated physiological saline solution (2 gm. of sodium citrate to each 100 cc. of normal saline). The tube should be corked and inverted gently 2 or 3 times to wash the cells, when it is placed in a centrifuge and revolved until the cells have been packed to the tip. The supernatant fluid is pipetted off to leave only cells, which are then diluted with normal salt solution to bring the fluid up to the 2 or 3 cc. mark (according to the amount of blood taken originally). The red cell suspension may be added to the tubes with a capillary pipette. By rinsing this pipette out with salt solution after use, it may be used for the control and any other unknown bloods to be tested at the same time to insure uniformity in the size of the drops. The results are recorded after the tubes have stood for an hour or two at room temperature. Reading from left to right, or from the higher numbered tubes to the lower, the tube showing just a slight tinge of color in the supernatant fluid due to hemolysis is taken as marking the point of initial hemolysis, while the first tube which shows no corpuscular residue on shaking, indicates the point of complete hemolysis. The percentage strength of the salt solution in the various tubes is easily calculated by multiplying the number on the tube by 0.02.

It is not necessary to have a predetermined normal standard with this method on account of the control which accompanies the test. Giffin and Sanford, however, have shown that normal blood shows beginning hemolysis in 0.42 per cent. to 0.38 per cent. and complete hemolysis with 0.36 per cent. to 0.32 per cent.

The method would seem to be of some value in the diagnosis of hemolytic jaundice, since the findings of all workers agree that there is an increased fragility, that is, that the cells hemolyze with higher strengths of salt solution. With pernicious anemia, with anemias of low color index, with splenic and with chronic obstructive jaundice, there is an increased resistance.

Demonstration of Bile Pigments in the Plasma.[1]—Blood is drawn from a vein to a test-tube containing potassium oxalate (see page 167). It is centrifugalized and the plasma removed with a pipette and placed in a small test-tube. Gmelin's test is used, the nitric acid being put under the plasma with a fine pipette. The plasma must be free from hemolysis. A white coagulum is formed at the junction and in the presence of bile a blue-green ring appears in the midst of the white zone. When pigment is small in amount, the color may not appear for a half-hour. Blankenhorn found that bile pigment was frequently found in pernicious anemia and was the cause of the jaundice. It does not appear in the urine because it is fixed in some way to the proteins of the plasma.

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[1] Gilbert's method, Compt. rend. Soc. de Biol., 1905.

THE PATHOLOGY OF THE BLOOD.

The Anemias.—The general term anemia implies an impairment in the quality of the blood. This may be manifested by a reduction in the number of erythrocytes or in the amount of hemoglobin or both. In considering the anemias we are confronted by a large number of clinical conditions between which it is not always possible to make sharp distinctions on purely hematological grounds. While many cases are so typical that they may be classified readily, a definite proportion remains in which differentiation is difficult because the blood picture is not clearly defined. In this field of diagnosis, as in all fields, the final decision (short of that based upon necropsy) rests in the clinic and a dogmatic opinion based upon the clinical laboratory findings alone often might be quite erroneous. Indeed, it is unfortunate that often final diagnosis must be postponed until postmortem pathological findings are available. This is illustrated in the discussion of pernicious anemia, where it will be shown that as conclusive as the microscopic findings in the blood may appear, they may be given in toto by the anemia due to dibothriocephalus infestation. This simply emphasizes the interdependence between laboratory and clinic, and justifies us in the following very brief consideration of the clinical features.

In attempting a classification of the anemias, we perforce adopt that which custom has made familiar, for while it is far from satisfactory, it fills working requirements fairly well. The anemias are divided into the so-called primary or essential anemias and the secondary anemias. In the latter the anemia is a mere symptom of the underlying disease which has produced it. The primary anemias are of unknown causation and with them the anemia itself predominates in the clinical picture.[1]

To simplify the understanding of the response of the blood-forming organs to the abnormal conditions which exist in anemias, we have found the following comparison helpful. The circulating system of the body may be likened to the highways and byways of a great battlefront. These roads are filled with volume of traffic of all sorts, similar to the blood in the fact that it is quite heterogeneous and made up of totally dissimilar constituents. The leukocytes may be likened to the soldiers; they are living objects, of different classes, serving different purposes, having different sources. Under abnormal circumstances in time of need, the reserves are called upon. For a time these are readily furnished, but when they are exhausted, the need for fighting material is so great that the homes (deposits of bone-marrow and lymphadenoid tissue) are drained for fighting power and immature soldiers are sent out, varying in character with the place of their origin (myeloblasts and myelocytes, and lymphoblasts).

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[1] An excellent discussion of the anemias from the modern point of view will be found in Minot's article in The Oxford Medicine, the Oxford University Press, New York and London, 1920, ii, 589.

The red cells, whose chief function is the transportation of oxygen may be likened to the motor transports, carrying supplies of a certain kind, ultimately wearing out, and being sent back to a base (the spleen) to be reduced to scrap. In cases of dire emergency, unfinished products may be sent out (nucleated red cells).

As with all animate objects, the "soldiers" have a certain duration of life. Some are lost on the various fronts (mouth, tonsils, bronchi, etc.). The blood, however, is not made up of these living cellular structures alone. They are carried in the current of traffic (plasma), which also carries the food material necessary to feed them and the civil population (the body's non-wandering cells), but also the waste products which may be sent back through the transport system to be gotten rid of at certain appointed stations (kidneys, intestines, lungs, etc.).

The Primary Anemias. Chlorosis (*Green-Sickness*).—Chlorosis, an anemia of simple type, is seen in either private or dispensary practice rather infrequently nowadays, possibly due to wide use of proprietary remedies containing iron. It is regarded as an exaggeration of the processes occuring at puberty where normally a change in the blood picture is seen as is evidenced by the drop in the erythrocyte count to which reference has been made. It is seen then between the ages of fifteen and twenty-five years. The diagnosis of chlorosis in mature life should not be made without convincing proof nor until other causative factors for secondary anemia have been definitely excluded. Its occurrence in the male is controversial. There is evidence that it has a tendency to afflict several members of a susceptible family. The onset is usually quite gradual. The color is not the chalky-white of post-hemorrhagic secondary anemia nor is it the lemon-yellow tint of pernicious anemia, but there is rather a sallow, muddy, pasty, edematous look which is especially marked around the eyes. Barker quotes McPhedran's warning about dismissing girls with especially red cheeks before examining the conjunctiva and tongue. There is no emaciation, indeed such patients are not infrequently plump. Among the manifestations which may be seen are digestive disturbances; notably perversions of appetite, tachycardia, blowing systolic precordial murmurs, nervous disturbances of the so-called neurasthenic type with alterations in disposition, headache, edema, amenorrhea and skin disorders, such as acne vulgaris. Recurrences may occur in later life.

The erythrocyte count may show little reduction, though counts of three to four million are customary. The total number of red cells in the body is not diminished, however, since it is generally agreed that the total amount of blood in the body is actually increased and apparent reduction is due to its dilution. The striking point about chlorotic blood is the low hemoglobin content. The hemoglobin content of the individual cells is reduced. This is made evident by the color index, which is of course merely the index of the hemoglobin content of the average cell as compared with normal. This may fall as low as 0.5. The white cells show no especial change. The platelets are often increased in number. In examining stained specimens one is at once struck with the red cells which present a greatly increased central pale area and sometimes appear as mere hoops or rings of hemoglobin. The cells may show slight polychromatophilia and slight anisocytosis. Nucleated red cells are not often seen, except in severe cases.

Diagnosis is comparatively easy when the age of the patient, the symptoms, the physical findings, and the blood pictures are viewed together. Certain diagnostic pitfalls should be avoided, such as early pregnancy, Basedow's disease, gastric ulcer, pulmonary tuberculosis, and nephritis.

Pernicious Anemia.—(**Synonyms.**—Addison-Biermer type of anemia, primary pernicious anemia, progressive pernicious anemia, Addisonian anemia, megaloblastic anemia, chronic hemolytic anemia of unknown origin.) Fundamentally, the fault is a destruction of erythrocytes by an unknown hemolytic factor. The bone-marrow attempts to compensate for this loss of red cells with more or less success for a time, but ultimately loses in the struggle.

The etiology is quite unknown. Anemia of this type progresses to a fatal termination, oftentimes with a number of remissions. It occurs in middle life or later and in contrast to chlorosis it affects both sexes about equally. The onset is usually gradual. The patient's first complaint may be of loss of strength, tiring easily, shortness of breath, palpitation, or other signs common to any anemia. Certain sensory symptoms, however, may predominate such as numbness of the extremities, tingling or other parasthesias, and awkwardness in handling small objects, and the initial symptoms may be those referable to disease of the posterior columns of the spinal cord, such as difficulty in locomotion, or retention or incontinence of urine. Dyspepsia or even vomiting is a frequent complaint, not to be wondered at when it is remembered that achlorhydria is almost constantly present. Intestinal symptoms too may be exceedingly difficult to combat, diarrhea being not unusual.

On physical examination we find the remarkable and typical color, a distinct lemon-yellow tint, which is sharply in contrast with the pasty appearance of the chlorotic. There is little if any wasting. Edema is occasionally seen. Hemic murmurs are heard in the precordial area and over the great vessels of the neck. Often ulcers of the tongue and buccal mucous membranes may be detected. The spleen is at times slightly enlarged. Signs of posterior or lateral column degeneration of the spinal cord are frequently encountered. Some elevation of temperature is ordinarily recorded.

Blood examination shows that the erythrocytes are markedly diminished in number, often to 1,500,000 or 2,000,000 even in ambulatory cases. Cabot directs attention to the fact that of 715 American cases, 150 did not consult a physician until the red count was below 1,000,000 cmm. In the terminal stages the count may drop to 150,000. The hemoglobin reduction, however, is quite disproportionate, for it drops slowly. Consequently the color index is high, practically invariably over 1.0, occasionally 1.5 (2.1 in one case). The white cells are diminished in number and ordinarily a relative lymphocytosis is present. This is understood when we remember that over-activity of megaloblastic tissue tends to exclude the leukocyte-forming centers from normal activity. The blood platelets, too, are markedly diminished in number. This finding may be explained by the deficiency of the bone-marrow in giant cells. The platelet count increases markedly above the normal after splenectomy, occasionally to 900,000 (Lee, Minot, and Vincent). The resistance of the red cells is greatly increased, and an increased proportion of reticulated erythrocytes are demonstrable by vital staining methods.

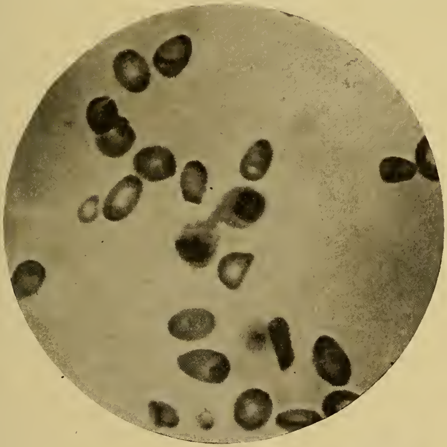

FIG. 22.—Blood in pernicious anemia, showing mitosis in circulating erythroblast.

It is considered (Lee, Minot and Vincent) that the presence of heavy knotted reticulum usually in the center of the cell is of particular importance in determining the activity of the bone-marrow. The specimens stained by Wright's or similar stains show a remarkable picture. The paucity of red cells is discerned, as is irregularity in size, shape, and staining (anisocytosis, poikilocytosis, and polychromatophilia). The red cells range in size from tiny microcytes to enormous macrocytes (2 or 3μ to 12 or 15μ in diameter) and vary in depth of color, when stained with Wright's stain, from the normal coppery-pink to a deep blue tinge. Under the staining irregularities should be included punctate basophilia (Fig. 22). The deformity of cell outline is especially noticeable. Knowing the color-index, one would predict the absence of central pale area, which is found to be almost universal, the cells being filled with hemoglobin. The occurrence of nucleated red cells is expected. These range from normoblasts to megaloblasts. Many hesitate to make a final diagnosis until megaloblasts have been demonstrated. To do this, it may be necessary to repeat the blood examination on several different occasions. The presence of nucleated red cells and of cells with marked polychromatophilia is evidence of the abnormal activity of the bone-marrow which, while unable to produce normal cells to replace those lost through some unknown destructive process, throws immature forms into the circulation. Methods of vital staining (q. v.) show the presence of reticulated red cells whose presence is similarly interpreted. Prothrombin is slightly reduced. The reduction is unimportant if active regeneration is in process. The urine contains large amounts of urobilin, due to the blood destruction.

At times blood examination may show large numbers of nucleated erythrocytes, so-called "normoblastic crisis," evidently representing a frantic effort on the part of the bone-marrow to supply new cells. After such crises, temporary improvement in the blood findings may follow. In certain of the more acute cases a neutrophilic leukocytosis may occur.

Remissions in this form of anemia are seen in a certain proportion of cases, during which the general condition is one of apparent health and the blood findings may simulate the normal. Of course a remission merely represents a pause in the downward course of the disease.

Aplastic Anemia.—The findings with aplastic anemia, clinical and otherwise, are similar to those of primary progressive anemia. Cabot's studies of 24 collected cases, shows that it is a disease of young persons, that it runs a rapid course with no intermissions, and that death usually occurs within a few months from onset of the disease. Subcutaneous hemorrhage and hemorrhage from the mucous membranes are extremely frequent. Fever is a marked symptom. From a hematological viewpoint, the chief point of differentiation is the lack of any signs whatever of attempts on the part of the bone-marrow to bring about regeneration. The blood findings are practically as described with the preceding similar anemia, except that nucleated cells are rare, reticulated cells are not seen and polychromatophilia, stippling, and poikilocytosis may be absent. Leukopenia is the rule, and the polymorphonuclear cells are much reduced in number. Platelets are far below normal, coagulation time may be prolonged only in occasional cases, and prothrombin is decreased. (See work of Musser, and of Drinker and Hurwirtz, of Cabot, and of Minot.) Post mortem shows that in the bone-marrow the erythroblastic tissue has disappeared, leaving bones filled with fat.

Secondary Anemias.—Anemias Due to Hemorrhage.—*After acute hemorrhages* there is a compensatory transudation of fluids from the tissues into the blood which is necessary to maintain the blood volume at a proper level. This addition of fluid dilutes the blood and accentuates the diminution in the number of red cells per cmm., the process continues for a short time after the hemorrhage has been arrested. When the hemorrhage has not been severe enough to produce death, we have prompt evidence in the blood findings of the fact that the bone-marrow is striving at regeneration. These may be briefly summarized as follows. The red cells are diminished though not as much so as the hemoglobin, since red cells are elaborated more rapidly, so that the color index is less than 1.0; the leukocytes are increased, especially the neutrophiles (which are of bone-marrow origin). The platelets due to the hyperplasia of the bone-marrow are present in greater numbers than normal. Occasional polychromatophilic red cells and normoblasts are seen (which indicate the release into the circulation of immature forms).

Chronic Anemias due to repeated hemorrhages or the continued destruction of erythrocytes arise from a variety of conditions, notably in hemophilia, scorbutus, infection with intestinal parasites, and hemorrhages from mucous membranes (gastric ulcer, epistaxis, hemorrhoids, etc.). The blood findings are similar to those in acute post-hemorrhagic anemia. The red count and hemoglobin percentage, however, may be reduced to lower levels and other changes may be of more severe type. There is slight leukocytosis. Some anisocytosis may be seen. With the intestinal parasites eosinophilia is found at times. Barker makes the point that when the hemorrhage occurs in such situations within the body that lost hemoglobin may be reabsorbed from the intestinal canal and may be used for the manufacture of new red cells, the color index is not so low and punctate basophilia of the reds is more pronounced.

Anemias Occurring in Malignancy and the Puerperium.—Anemias due to these causes are ordinarily of the type seen in chronic hemorrhages, but occasional exceptional instances of anemia make the recognition of these possible causative factors all the more important. With cancer we have to do with two factors, the loss of blood by hemorrhage and the destruction of blood by hemolytic substances. In cases where the new growth is situated in the digestive tract, an added factor may be starvation due to interference with the digestive process. The anemia in sarcoma cases is frequently extremely severe, and that due to the invasion of the bone-marrow by metastases of malignant tissue are particularly profound. There are instances where the color index is not markedly lowered and where the blood findings consequently verge upon those seen in pernicious anemia.

Anemias Resulting from Infections.—Anemia is seen with practically all acute febrile conditions. It is likely to be especially marked with acute articular rheumatism, typhoid fever, malaria, acute septic conditions, chronic suppurations, syphilis and tuberculosis. The factors may be the actual destruction of erythrocytes by the infecting organism (*e. g.*, streptococcus hemolyticus, malarial plasmodium, etc.), to the interference with nutrition due to limitation of diet or to actual loss of blood by hemorrhage. In these cases the anemia is usually of the so-called "secondary" type just described.

Anemia is seen in certain cases infected by the dibothriocephalus latus. The anemia may be of the chlorotic or secondary type with low color index or it may be indistinguishable from pernicious anemia, showing high color index, poikilocytosis, polychromatophilia, punctate basophilia, and nucleated red cells, including even megaloblasts. The liberation in the intestinal tract by decomposed segments of the worm of a toxic substance and its absorption by the host is held by some (Tallquist and Faust) to produce the blood destruction.

Anemia Due to Intoxications.—Anemia may be produced by certain inorganic poisons, such as mercury, lead and arsenic, by autogenous poisons generated in chronic affections, such as nephritis and jaundice, and by benzol.

Of especial interest is the *anemia produced by benzol* because of the trial which it was given as a therapeutic agent in myeloid leukemia and because of the poisoning resulting from its use in various industries, especially where rubber is handled. The problem of its effect upon the hematopoietic organs has been exhaustively studied by Selling. The clinical features presented are a purpura hemorrhagica with skin lesions, retinal hemorrhages and bleeding from the mucous membranes. The symptoms of the poisoned cases are those of a marked toxemia, with high fever and rapid pulse and before death in fatal cases delirium (Harrington). The anemia is of the aplastic type, the red cells reaching 640,000 in one case (Selling), and 1,150,000 in another. The red cells show only slight changes, chiefly anisocytosis, without poikilocytosis. There is little evidence of regeneration as shown by the absence of nucleated forms. Blood platelets are scanty. There is a decided leukopenia, counts as low as 140 before death being reported (Selling). The polymorphonuclear percentage is greatly decreased and the mononuclear cells relatively increased.

The **anemia produced by lead poisoning** is also of practical interest on account of the frequency of plumbism in the trades, notably among those who handle lead paint, in battery-makers, metal-polishers, and compositors. The red corpuscles fall to three and a half million or less, and the hemoglobin falls more or less in proportion. There may be a slight leukocytosis or a slight leukopenia. The striking feature is the presence of basophilic stippling of the red cells, so-called punctate basophilia. While this has been regarded by some as pathognomonic of lead poisoning, it is not, since it is found in other conditions, notably pernicious anemia or the more severe secondary anemias. Some writers have maintained that the presence of 100 basophilic red cells per million corpuscles should lead the factory physician to watch the worker's health closely, and the presence of 300 per million to cause his suspension from work. There are many definite cases of plumbism, however, which do not show basophilic granulation of the red cells, and its absence in a doubtful case is not to be considered as excluding lead poisoning from diagnostic consideration. Granulation may appear promptly after the ingestion of lead. White and Pepper showed it might be found in the peripheral blood twenty-five hours after the ingestion of 7.5 grains of lead acetate.

Anemias Due to Poor Hygienic Surroundings (Anemia of the poor).—This type is frequently encountered in hospital and dispensary practice. Overcrowding and the consequent lack of fresh air, the deficiency of food, particularly of varieties rich in iron, and overwork are the factors which lead to inanition and subsequently deficient production of blood, producing a mild or moderate type of secondary anemia.

Leukanemia.—This term is rapidly passing into disuse since it is felt that most cases whose blood pictures combine the features of leukemia and pernicious anemia are either leukemia with terminal anemia or pernicious anemia with lymphoid marrow.

Other Anemias Showing Splenomegaly.—In addition to the anemias already described, there are certain syndromes presenting, among other features, splenic enlargement and secondary anemia. Brief consideration of them is in order, simply because of the anemia. The literature on this subject has been carefully studied by Pearce and co-workers. The table presented on page 83 from their work gives a resumé of the conditions in which splenomegaly and anemia are coexistent.

Gaucher's Disease.—The onset is in infancy or childhood and is insidious. The course is chronic, usually about twenty years. Later there is distinct anemia, subcutaneous and submucous hemorrhages, and progressive enlargement of the spleen. There is nothing characteristic about the anemia. It is of the chlorotic type, with definite leukopenia.

Banti's Disease.—It usually occurs in young and previously healthy adults. The course is chronic. First there is a period marked by increasing pallor, weakness, digestive disturbances and abdominal pain. It may be noted now that the spleen is enlarged, smooth, and hard. A tendency to hemorrhages is seen. The anemia is moderate, of the chlorotic type, with no distinguishing characteristics. The resistance of the red cells is normal. Leukopenia is the rule.

Later, there is a slight increase in the size of the liver; the urine is small in amount, highly colored, and contains much urobilin. Diarrhea and dypsepsia become bothersome. Finally symptoms of liver cirrhosis predominate the picture, there is more or less ascites, slight jaundice, increasing anemia, and emaciation. Death is by hemorrhage or intercurrent infection.

Anemia Pseudoleukæmica Infantum (*v. Jaksch's Disease*).—This condition is occasionally encountered in infancy and early childhood.

Exact classification is difficult, since the same term is used to include many different pictures and probably should not be employed at all. In fact, like the term leukanemia, it is rapidly disappearing from current literature. Attention has been directed to the prompt response of infantile bone-marrow to any stimulus. One would therefore expect to find in a variety of conditions nucleated red cells and myelocytes in the circulation under much lesser provocation than is required to bring them out in adult life.

In cases which have been described as V. Jaksch's disease, the spleen is large, smooth, and hard. The red cells are much reduced in number, possibly as low as one million. The hemoglobin is not reduced proportionately, so the color index is over 1.0. There is marked anisocytosis, polychromatophilia, and punctate basophilia, and many nucleated red cells both of normoblastic and megaloblastic type. The leukocytes are usually increased in number, from 20,000 to 100,000 per cmm., the neutrophilic leukocytes predominating. Myelocytes may be seen. The blood findings therefore are at first quite confusing. It is stated that the condition may develop into anemia, into myeloid leukemia, or terminate ultimately in recovery. Doubtless this simply means that the condition was originally a more or less severe anemia or a leukemia, that the blood picture was confused for the reasons already stated, and that time was required for the typical picture to appear. In interpreting the results of blood examination in infants and children, it is always well to remember the readiness with which the bone-marrow responds to stimuli and to make suitable allowances in evaluating seemingly grave findings.

Hemolytic Jaundice.—1. *The acquired type of hemolytic jaundice* (Hayem-Widal) is characterized by chronic jaundice with the presence of bile pigment in the blood serum though not in the urine, and by splenic enlargement. There are no signs of obstructive jaundice, such as clay-colored stools and itching. The spleen is large and hard, the liver possibly slightly enlarged. The anemia may be sufficiently grave to dominate the clinical picture. The blood picture is often similar to that seen in pernicious anemia, with high color index, anisocytosis, and poikilocytosis. The leukocytes, however, may be increased in number. (For discussions of hemolytic jaundice see the reports of Thayer and of Tileston.) Counts below 1,000,000 are recorded in cases (Pearce). Increased numbers of microcytes and of reticulated red cells have been reported, pointing to the attempt at regeneration. The red cells show *decreased* resistance to hypotonic salt solution. There are periods of exacerbation, termed by Widal "crises of deglobulization" in which the jaundiced becomes more intense and bile appears in the urine.

The exact pathogenesis is the subject of debate, some holding that the fundamental fault is in the blood cells (a dystrophy of the blood cells), others that there the spleen has an exaggerated hemolytic activity. Marked improvement follows splenectomy according to Pearce, Krumbhaar, and Frazier.

THE SPLEEN AND ANEMIA.*

	Etiology.	Pathology of spleen.	Family history.	Time of onset.	Duration.	Icterus.	Splenomegaly.	Hemorrhage.	Anemia.	Resistance of red blood cells.	Reticulated red blood cells.	Urobilinuria.	Leukocytes in blood.	Liver.	Treatment.
Gaucher's disease	Toxin (?) infection (?) tumor (?)	Peculiar cell hyperplasia	Occasionally positive	Childhood	Many years	Rare	+++	Occasionally	Slight	?	?	?	–	+	Splenectomy.
Banti's disease	Toxin (?) infection (?)	Hyperplasia fibrosis	Negative	Adult life	Few years	Rare	+	Occasionally	Slight to severe	Normal	Normal	+	–	First + Later –	Splenectomy.
v. Jaksch's disease	Rachitis (?)	Hyperplasia fibrosis	Negative	Infancy	Several months	Rare	++	?	Slight to severe	?	?	?	+	+	Splenectomy, iron, arsenic, etc.
Hayem-Widal[1]	1, primary(?); 2, secondary to infection	Congestion and pigmentation	Negative	Any age	Many years	Present	++	Rare	Severe	Diminished	Increased	+	–	+ or normal	Splenectomy.
Chauffard-Minkowski[2]	Heredity	Congestion and pigmentation	Positive	Congenital or childhood	Many years	Present	++	0	Slight	Diminished	Increased	+	–	+	Splenectomy.
Pernicious anemia	Enterogenous toxins (?)	Fibrosis	Negative	Adult life	Few years with remissions	Very rare	Slight or diminished.	Rare	Severe	Increased	Increased	+	– or +	Normal	Iron, arsenic, etc. (splenectomy questionable).

* From "The Spleen and Anemia," by Pearce, Krumbhaar and Frazier. J. B. Lippincott & Co., Philadelphia, 1918, p. 244.

[1] Acquired hemolytic jaundice.

[2] Congenital or familial hemolytic jaundice.

2. *The familial type of hemolytic jaundice* (Chauffard-Minkowski) is quite similar to that just described, but there is a history of other cases in the family and of symptoms dating from early life. The spleen is enlarged but certain clinical differences are seen. The acquired form usually starts with an acute illness and the patient is anemic rather than jaundiced. In the familial type, jaundice may be noted but it may be lightly regarded by the patient and dismissed by him as a family characteristic which does not interfere with his health.

The blood picture is not usually as severe as that of the acquired type, the average count of 104 cases being 3,340,000, ranging from 1,800,000 to 5,700,000. When severe, it may be indistinguishable from pernicious anemia.

The Leukemias.—In leukemia there is extensive permanent hyperactivity of the tissue producing leukocytes with escape of an excess of cells into the circulation. The transitory leukocytoses accompanying certain infectious diseases are not included in this heading. Either the lymphatic or the myeloid tissue may be involved, and the condition is accordingly referred to as lymphatic (lymphoblastic) or myelogenous (myeloid, myelocytic, splenomyelogenous) leukemia, respectively. In either case the condition may be acute or chronic, the acute cases being relatively rare.[1]

Chronic Myelogenous Leukemia.—The onset is usually insidious, the patient noticing first the abdominal tumor, or abdominal enlargement due to the increased size of the spleen, though gastric-intestinal symptoms, shortness of breath, or some symptoms due to anemia may be prominent early complaints. The blood is fairly characteristic. Its whiteness may be discerned grossly, and the preparation of satisfactory films is often difficult because of its creamy consistence which makes the drop spread slowly. The leukocyte count is usually high, occasionally reaching 1,000,000 per cmm. (Osler). Frequently an anemia of the secondary type is seen, which may range in intensity from slight to extremely severe. This may be accounted for by the hyperplasia of the myeloid marrow tissue, which crowds out erythrogenic tissue. The stained specimens give a striking picture, the leukocytes occasionally equalling or even exceeding the red cells in number. All types of leukocytes show an absolute increase. The predominant feature is the presence of myelocytes (see preceding description), or of myeloblasts, (immature non-granular myelocytes) which may comprise 30 to 50 per cent. of the total number of leukocytes. Nucleated red cells are seen; usually they are normoblasts, though megaloblasts are not infrequently seen. The blood picture is subject to considerable variation, since most cases have a course marked by many ups and downs. During remissions, especially after x-ray or radium treatment, the blood findings may be practically normal, in regard to both the leukocyte count and the differential formula. During the course of acute infections, the differential count may show a preponderance of neutrophilic polynuclears.

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[1] The recent article by Ordway and Thomas should be consulted by the student. It will be found The Oxford Medicine, the Oxford University Press, New York and London, 1920, ii, 681.

Chronic Lymphatic Leukemia.—Ordinarily the first symptom noted is an enlargement of the lymphatic glands, usually beginning in the cervical region. There is often a history of trauma. Some splenic enlargement may be seen and an anemia of the secondary type develops. The lymphatic enlargement may be present for some time before a diagnosis is made for the truth may be revealed only by a routine blood examination.

The leukocyte count averages from 100,000 to 500,000 per cmm., ranging from less than 10,000 to over 1,000,000. Cabot calls attention to the large proportion of low or moderately low counts in his series, comparable to the leukocytosis of infectious disease, and to the likelihood of such counts being overlooked in a hasty examination. Stained films show the predominance of lymphocytes, which make up 90 per cent. or more of the total number of leukocytes. Ordinarily these correspond to the small mononuclears or small lymphocytes of the normal blood, but occasionally there may be lymphoblasts and many lymphocytes, the latter identical in all respects with the small lymphocytes except for their greater size.

As with myeloid leukemia, the course of the disease may show many remissions during which the blood picture varies, though there is ultimate progression to a fatal outcome. The oxydase reaction is of assistance in doubtful cases in demonstrating that the mononuclear cells have no oxydase granules and therefore, are not myeloid cells.

Acute Myelogenous Leukemia.—This condition is relatively rare. It is marked by a fulminating, febrile course, advancing rapidly to fatal termination. The blood picture may be similar to that of chronic cases. Myeloblasts may predominate, however, and the granular myelocytes may be absent or few in number, so that confusion may arise, and recourse to special staining methods (see oxydase reaction) may be necessary to determine the origin of these non-granular leukocytes and so to establish the fact that one is dealing with acute myeloid leukemia rather than with acute lymphoid leukemia.

Acute Lymphatic Leukemia.—The onset is usually sudden, marked by throat symptoms such as difficulty in swallowing, swollen gums, high temperature, hemorrhagic signs, and later by swelling of the glands. The white count increases rapidly up to 500,000 per cmm. (Barker). The cells are often chiefly of the large lymphatic type. There is an early fatal termination.

THE CLINICAL SIGNIFICANCE OF THE FINDINGS OF BLOOD EXAMINATIONS.

The interpretation of the findings is a matter of judgment, which must have as its basis clinical experience. Indeed diagnosis of the anemias often rests as much upon clinical grounds as upon consideration of the laboratory findings. These have been considered rather fully under the appropriate headings.

As may be seen from the discussions in the preceding sections, many factors influence both the actual number of leukocytes and also their relative proportions. Many of the findings taken by themselves are of little moment, since in general, any bit of information, clinical or laboratory, is valuable in inverse proportion to its frequency. For example, polymorphonuclear neutrophilic leukocytosis is found in a variety of conditions so that a correspondingly large list of possible diagnoses may be considered when it is found. Eosinophilia, on the other hand, is seen less often. Its occurrence leads often to the correct conclusion with promptness. In practically no instance, however, except with parasitic blood diseases, do we have findings which are absolute. Almost invariably, they must be taken as part of the clinical evidence and weighed as such.

Leukopenia in a febrile condition suggests the possibility of typhoid fever, malaria, or tuberculosis infection. In the early stages of the *exanthemata*, a differential count may be of service; for example, in differentiating between *measles and scarlet fever*. Typically, measles shows a lymphocytosis and no eosinophilia; scarlet fever manifests a polymorphonuclear leukocytosis and often an eosinophilia.

When *typhoid fever* is suspected, leukopenia is of significance in differentiating from other acute infections except tuberculosis or malaria. During the first ten days blood cultures (q. v.) may serve to establish the diagnosis while later, agglutination tests (q. v.) are of more value. During the course of a typhoid infection it is worth while to make a count of the white blood cells at stated intervals, say twice weekly, to determine the level which the count maintains, since symptoms of perforation or hemorrhage may make a count desirable and interpretation would be more reasonable when based on a knowledge of preëxisting conditions.

In *septicemia*, there is usually a leukocytosis with increase in the number of polymorphonuclear cells. Blood cultures (q. v.) frequently reveal the infecting organism.

Cases of pulmonary tuberculosis with few exceptions show a chloro-anemia, characterized by a normal or slightly subnormal number of erythrocytes with a markedly decreased percentage of hemoglobin. With a moderate or slight degree of involvement there is no increase in the number of leukocytes. The more advanced cases show a leukocytosis of only slight degree, unless there are complications. In the earlier stages and with a small degree of involvement, the percentage of lymphocytes is higher and that of polymorphonuclears smaller while the more advanced and unfavorable cases show a lower percentage of polymorphonuclears, according to Craig and M. Solis-Cohen and Strickler. This checks well with the fact that the lymphocytes play a definitely protective role in tuberculosis.

In regard to the interpretation of a leukocytosis, it should be remembered that leukocytosis is largely dependent upon resistance of the body against infection, while the increase in the proportion of polymorphonuclear cells in the differential count is taken as an index to the severity of the infection. (Sondern.)

Generally, therefore, a sharp leukocytosis is a hopeful sign in those conditions in which it is ordinarily expected; conversely, its absence in such instances is of poor prognostic import. For example, a pneumonia patient with a count of 20,000 or 25,000 does not cause the attending physician the anxiety that one would with a count of 8,000, others conditions being equal.

Sondern has drawn the following conclusions from a study of counts in *pneumonia cases:*

1. Slightly increased polynuclear percentage indicates slight toxic infection, regardless of the leukocyte count.
2. Greatly increased polynuclear percentage indicates severe toxic infection, irrespective of the leukocyte count.
3. Slight leukocytosis with slight polynuclear increase indicates fair resistance and slight toxic infection.
4. Pronounced leukocytosis with slight polynuclear increase indicates good resistance and a slight toxic infection.
5. Slight leukocytosis with pronounced polynuclear increase indicates poor resistance and a severe toxic infection.
6. Pronounced leukocytosis with pronounced polynuclear increase indicates good resistance and a severe toxic infection.
7. Absence of leukocytosis with pronounced polynuclear increase indicates no resistance and severe toxic infection, and a falling leukocytosis with a rising polynuclear percentage indicates diminishing resistance and increasing toxic infection.
8. Falling leukocytosis with a falling polynuclear percentage indicates diminishing toxic infection or recovery.

It has been definitely shown that the mortality in pneumonia cases with a leukocyte count below 10,000 is 55 to 60 per cent. and that with a rise in the leukocyte count from 10,000 to 30,000 there is a progressive decrease in the mortality (Chatard, Avery, Chickering, Cole, and Dochez). When consolidation of the lobe or lobes is taking place, or during the period when a spread of infection to a new area of lung is in progress, there may be a well marked decrease in the number of leukocytes, even to a point below 10,000. A steady rise in the leukocyte count is usually of good import. A continuance of a high count even after crisis, especially if associated with a high percentage of polymorphonuclear cells, suggests some complication with pus formation. Continued leukocytosis with diminishing polymorphonuclear count, correspondingly increased lymphocyte count, and the presence of a few eosinophiles, suggests delayed resolution, or serum sickness, if the patient has been treated with specific serum. (Avery, Chickering, Cole, and Dochez.)

Accepting Sondern's hypotheses regarding the significance of increased total leukocyte count and increased polymorphonuclear percentage, Gibson has advanced a theory which is the basis for a curve, arranged to show the disproportion between total leukocyte increase and increase of polymorphonuclear percentage. He assumes as standard a leukocyte count of 10,000 and a percentage of polymorphonuclear cells of 75. Paper ruled in squares is used for plotting and the horizontal base line is taken as representing a count of 10,000 white cells and likewise an increase of 1 per cent. in the polymorphonuclear cell count. The white count is plotted on the left hand side of the sheet and the percentage of polymorphonuclear cells on the right hand side. A line is drawn from point to point. When the proportion is normal, the line connecting the two points is assumed to be approximately horizontal. A rising line is interpreted as indicating a rather more severe lesion and less resistance, and if the interval between the two points is considerable, for example, ten squares or more, this fact is taken as indicating a severe lesion. A line horizontal or approximately so, indicates that the lesion, even if severe, is well borne.

Certain writers claim to have found the Gibson curve useful, but have objected to the complexity of lines which the chartering of continued observations would entail. Walker has suggested the use of an index, since the "resistance line" is simply the visual expression of the disproportion between the total leukocyte count and the polymorphonuclear percentage. He assumes that the same arbitrary standard for the white normal that Gibson does and takes 70 normal percentage for polynuclear cells. The formula is:

$$(\mathrm{T}-10)-(\mathrm{P}-70)=\mathrm{I.R.}$$

T is the digits in the thousandths place of a given leukocyte count; 10 is the digits in the thousandths place of the high normal leukocyte count; P is the percentage of polymorphonuclear cells in the differential count of the same patient at the same time; 70 is the high normal polymorphonuclear percentage; and I.R. represents the "index of resistance." As an example, if the total leukocyte count were 15,000 and the polymorphonuclear percentage 80, substitution in the formula would give $(15-10)-(80-70)=-5=\mathrm{I.R.}$ This is a negative index. indicating according to the theory a severe infection which is comparatively greater than the resistance of the body to it and corresponds to a rising line on the Gibson chart. If, however, the total leukocyte count is 25,000 and the percentage of polymorphonuclear cells 75, substitution in the formula would make the index of resistance $+10$, a positive index, corresponding to a falling resistance line, and pointing to a good reaction to a comparatively mild infection. The use of the index of resistance in this manner makes transference of the results to graphic charts a simple matter. Enough work with the Gibson curve has not been reported to make it possible to determine finally its clinical value.

In surgical conditions, Sondern has defined three distinct pictures: First, a relative percentage of polymorphonuclear cells below 70 per cent. With an inflammatory leukocytosis of any degree, he feels that this excludes the presence of pus or gangrene and points to good body resistance against infection. Second, an increased relative proportion of polymorphonuclear cells with little or no inflammatory leukocytosis is an absolute indication of the inflammatory process. The percentage is a direct guide to the severity of the infection. In adults, a purulent exudate or gangrene is uncommon with a polymorphonuclear percentage below 80 per cent. and a percentage over 85 per cent. is rare without purulent exudate or gangrene. In children, the percentage of polymorphonuclears is less than with adults and pus is sometimes seen with a percentage as low as 73. Third, an increased percentage of polymorphonuclear cells and a decided leukocytosis. Most of the cases of inflammatory lesions fall into this class. Here the percentage of polymorphonuclear cells is held to be a guide to the status of the inflammatory lesion. In following such cases, the Gibson curve (q. v.) might serve to present the changes from time to time in a graphic way.

The Arneth Formula.—Arneth has described very fully the possible variations in the nuclear arrangement of the polymorphonuclear neutrophilic leukocytes. He has divided them into five groups according to the number of nuclear lobes, those in Group I have one nuclear lobe, those in Group II have two nuclear lobes, etc. In the first group are placed the mononuclear forms, subdividing this into (*a*) those with a single unindented nucleus, corresponding to Ehrlich's myelocytes, (*b*) those with but a slight indentation of the nucleus (metamyelocytes), (*c*) those which the indentation has extended further than in the preceding group but is not sufficient to separate it into two really distinct portions. Cells of the first types, *a* and *b*, are not seen in healthy subjects while those of the type *c* normally compose about 5 per cent. of the neutrophiles.

The second group, comprising cells with two distinct nuclear segments, constitutes in normal cases about 35 per cent. of the total neutrophiles. Cells of the third group, characterized by three nuclear portions, are usually present to the extent of about 41 per cent. of the total neutrophiles, while cells, those of the fourth group, having four nuclear fragments, ordinarily form 17 per cent. of the neutrophiles. Cells of the fifth group have five or more nuclear subdivisions and normally form about 2 per cent. of the total. It has been assumed that cells pass through various stages in the process of development, that the cells of the first group are the youngest in the point of development and those of group V the most mature, the other groups representing intermediate stages. Arneth's clinical deductions have not received universal support.

In recording the results of the count the following scheme is usually employed:

Group No.	I.	II.	III.	IV.	V.
Percentages	5	35	41	17	2

To make graphic representation of the results possible, Arneth took the sum of the cells in the first two groups as the index. In this country the index proposed by Bushnell and Treuholtz is more commonly used. This takes as the index the sum of the cells in the first and second groups and one-half of those in the third group. There is some disagreement as to the exact index for normal individuals, this fact pointing to the influence of personal equation in classifying the nuclear divisions. The normals obtained by various workers are Arneth, 60.5 (when his figures are translated to terms of Bushnell and Treuholtz's standard); Briggs, 57.73; Bushnell and Treuholtz, 67; Miller and Reed, 48, with a normal range from 45 to 55; Minor and Ringer, 48.5. An increase in the index is spoken of as a "shift to the left," meaning of course, an increase in the number of cells in the first, second and third groups. By many workers, this "shift to the left" is interpreted as indicative of low resistance in tuberculosis. They hold that there is a return of the cells in the groups to the right is found with improvement. This point, however, is not universally accepted, and is opposed by Cohen and Strickler.

Döhle Inclusion Granules.—Döhle has described the occurrence in the polymorphonuclear neutrophiles of small coccus-like or rod-shaped bodies, usually situated at the periphery of the cell. While they may be demonstrated with Wright's or Giemsa's stain, it is stated by some workers that the use of pyronin-methyl-green is preferable. With this stain, the Döhle bodies stain bright red and the nuclei of the cells purple. While the bodies appear to be present in scarlet fever with considerable constancy, they are also found in other conditions. The absence in suspicious cases rather tends to exclude scarlet fever, but their presence can not be taken as conclusive confirmatory evidence.

For demonstration of the bodies, Pappenheim's methyl-green and pyronin or Granger and Pole's modification of Manson's stain may be employed. The former is prepared by mixing two parts of a saturated aqueous solution of pyronin with four parts of a saturated aqueous solution of methyl-green. With this stain the inclusion bodies stain bright red and the nuclei a greenish-blue or purple. Granger and Pole's modification of Manson's stain is made as follows: Methylene blue, 1.5 gm.; absolute alcohol, 10 cc.; 5 per cent. solution of phenol in water, 100 cc. In either case, fixation should be accomplished by flooding the slide or cover-slip with absolute methyl alcohol, which is allowed to remain for two minutes. The nuclei and the inclusions stain a deep blue and the cytoplasm a pale blue with this stain. While the bodies appear to be present with considerable frequency in scarlet fever, especially up to the fourth day and even to the tenth day, they are also found in diphtheria, pneumonia, tuberculosis, sepsis, and severe streptococcal angina. They are not found in serum rashes or in toxic rashes due to other causes than serum, nor have they been found in German measles. Definite cases of measles from the preeruptive stage through the first week do not show a picture similar to

EXPLANATION OF PLATE IV.

(*Park and Williams' Pathogenic Microörganisms*).

Partly schematic. Drawn and rearranged by Williams, partly from Muir and Ritchie, partly from Kolle and Hetsch and partly original. Giemsa's stain.

The asexual forms show cycle of the organism in the red blood cells of the human host. They show schematically the time of fever and the day of segmentation.

Tertian type.

Fig. 1.—Segmented organism.

Fig. 2.—Young ring form in cell and a young form on surface.

Fig. 3.—Growing schizont; irregular form due to great motility; beginning pigment formation; red blood cell becoming paler.

Fig. 4.—Larger schizont with dividing nucleus. Red blood cells pale and stippled.

Fig. 5.—Nucleus divided into four clumps.

Fig. 6.—Further division of chromatin and formation of irregular rosette. Pigment finely granular in center.

Fig. 7.—Segmentation. Note eighteen merozoites (usually sixteen).

Quartan type. Shows following differences from tertian. Slightly larger, fewer segments (usually eight), and more regular. Pigment coarse. Red blood cells unaltered. Segmentation every seventy-two hours.

Estivo-autumnal type. Shows following differential points: Merozoites smaller and more numerous (thirty-two?); organism less motile with less pigment. Red blood cells smaller and greenish color (in fresh cells).

Sexual Forms. Show cycle of development in mosquito.

Fig. 1 (*A* to *E*).—Male (♂) and female (♀) forms of tertian type formed in human blood; *F*, flagellation of male type in stomach of mosquito; *G*, *H*, changes in female type and fertilization in stomach of mosquito.

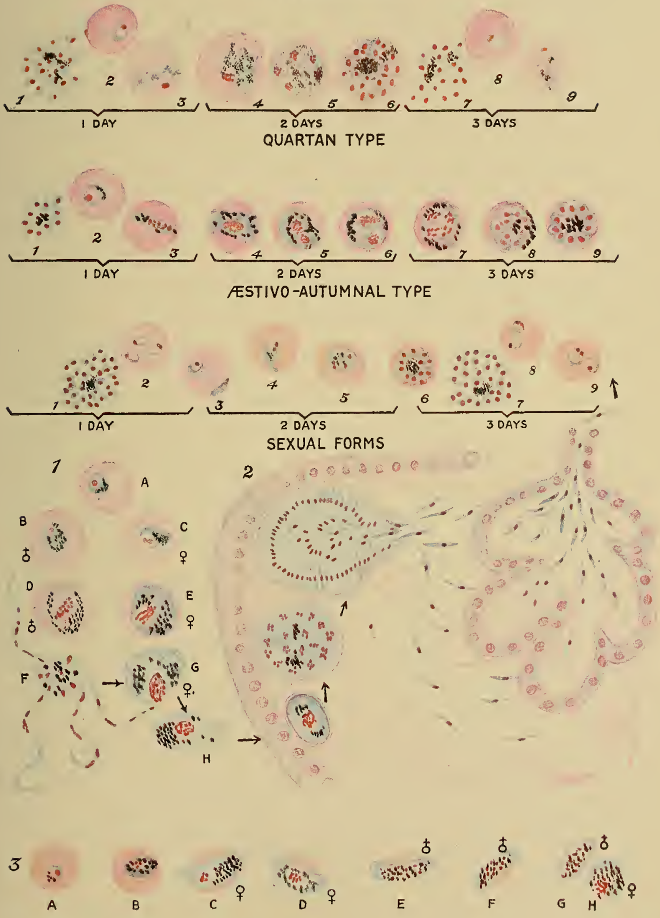
Fig. 2.—Development of sporocyst within mosquito. Liberation of sporozoites, which find their way to the salivary gland.

Fig. 3.—Sexual forms of estivo-autumnal type found in human blood, showing development of sickle-shaped bodies.

PLATE IV

ASEXUAL FORMS OF MALARIAL PARASITES
(SCHIZOGONY)

TERTIAN TYPE



A. W. WILLIAMS, DEL.

scarlet fever, though tiny granules are found in the polymorphonuclear quite unlike the larger granules seen in scarlet fever. From the work which has been done by Kolmer and Nicoll and Hill it would appear that the absence of the bodies in suspicious cases tends to exclude scarlet fever but that their occurrence cannot be taken as conclusive evidence of its presence.

DISEASES IN WHICH THE BLOOD CONTAINS PARASITES.

Malaria.—In malarial infection, man serves as the intermediary host for an infection by one of three forms of plasmodium, the definitive host for the infecting organism being the female Anopheles mosquito. The plasmodium may be of three species, P. vivax, P. malariæ, and P. falciparum giving rise respectively to the so-called benign tertian, quartan, and estivo-autumnal clinical varieties of the malaria. In man, only the asexual cycle of development takes place, and the sexual forms which are seen in the circulating blood are simply the gametes which develop from the asexual parasites, and whose development goes no further in the human host.

Asexual development proceeds by geometrical progression after the entrance into the circulation of the first sporozoite, but ordinarily there are not enough parasites to produce a paroxysm until about two weeks after infection. The paroxysm takes place at the time when the schizont (presegmenting forms) rupture and the paroxysm is thought to be due to a toxin set free by the rupture of the schizont.

In examining blood for malaria, fresh unstained specimens may be employed. This method has the advantage of permitting observation of ameboid movement; but it has the disadvantages that the examination must be carried out practically at the bedside and that recognition of the unstained parasites is not especially easy unless one has had much practice with this particular method. Therefore, the use of a suitable stain is recommended. Wright's stain is most useful as it stains the chromatin and is well adapted to show all the details of the parasite.

When the clinical signs give rise to the suspicion of malaria, quinine should be withheld until the blood examination has been made, because the ingestion of doses of this drug, quite insufficient to influence the course of the infection, will often cause a disappearance of the parasites from the circulating blood, for the young merozoites may be killed as soon as segmentation takes place, or the shape and staining reaction may be materially altered.

Occasionally it is impossible to demonstrate parasites in the ordinary blood films after the most painstaking search, even when quinine has been withheld. In such instances, concentration of the blood film may be employed.

According to the Ross-Ruge method, a very thick smear is made on a slide. After drying, the slide is treated with the following fluid:

Formalin	5 cc.
Acetic acid	1 cc.
Distilled water, to make	100 cc.

This serves to fix the smear and at the same time to take out the hemoglobin. The slides should be washed with water, dried and stained with Wright's stain, followed by treatment with borax-methyllene blue (borax, 2.5 gm.; methylene blue, 1 gm.; distilled water, 50 cc.).

This method is being supplanted by the concentration method of Bass and Johns: 10 cc. of blood is obtained by venipuncture and placed in a tube containing 0.2 cc. of a solution of glucose and sodium citrate (sodium citrate, 50 gm.; dextrose, 50 gm.; dissolved by heat with sufficient water to bring volume to 100 cc.). The blood is mixed well and divided between two centrifuge tubes (preferably round bottom tubes 15 cm. inside by 12 cm. long), placed in Cornell shields. These tubes are centrifugalized at about 2500 revolutions per minute, long enough to bring the parasites and leukocytes to the top layer of the sediment and still not long enough to pack the corpuscles. It has been determined by Bass and Johns that if the radius of the centrifuge be 18 cm., the time should be one minute for each cm. of blood column. At the end of the proper time interval, the top layer of sediment should contain all the parasites and the leukocytes. A capillary pipette is used to skim off this 1 mm. layer with an equal quantity of serum and to place the mixture in a tube about 12 cm. long and 0.5 cm. bore. The tubes may be made from glass tubing of suitable size. Should the column exceed 5 cm., the mixture should be divided between two tubes. The tubes are again centrifugalized, the top layer of sediment is drawn into a large capillary pipette to form a column not over 5 cm. in height which is mixed by forcing in and out on a slide, and is finally drawn back up into the capillary in such a position that the end may be sealed in the flame. The capillary is cut with a file above the blood column and carefully packed with a little cotton into a centrifuge tube so that it may be centrifugalized. When this has been done the gray leukocyte layer at the top of the sediment with the cells directly underneath should be removed with a fine capillary pipette, placed on a slide, mixed, smeared in the usual way, and stained with Wright's stain.

Development of the Parasite.—The understanding of the blood picture in malaria is simplified by knowledge of the life history of the infecting parasite. The organisms belong to the order hemosporidia, subclass telosporidia of the class protozoa. Ordinarily the infection of man takes place through the bite of an infected female Anopheles mosquito, which discharges through the veneno-salivary duct in the hypopharynx the sporozoites into the circulation of the human upon whose blood she is feeding. In this human intermediate host only the asexual cycle of the development (schizogony) takes place. The sporozoite enters a red cell, grows into a schizont, when division of the chromatin takes place with the formation of a number of merozoites. These are liberated coincidently with the chill and infect a corresponding number of red cells. Each of these newly formed bodies repeat this cycle, and the multiplication of the parasites proceeds by geometrical progression.[1]

The sexual cycle (sporogony) takes place in the body of the Anopheles mosquito. When the mosquito has ingested blood from an infected human, the gametes are taken into the stomach and the macrogametocyte (female form) goes through the process of nuclear reduction with the formation of polar bodies. The microgametocytes (male form) extend rapidly motile flagellar projections which are broken off and become microgametes. One of these enters a macrogamete and fertilizes it, an oökinete or vermicule resulting. This penetrates the wall of the stomach by a boring motion and lodges just under the outer layer of the midgut, where development proceeds into a large zygote or oöcyst. The oöcyst has a capsule and contains numerous small nuclear bodies. Each of these spherical bodies (sporoblasts) contains chromatin-bearing slender rods. The oöcyst ultimately ruptures into the cœlomic cavity where the rod-like bodies (sporozoites) are discharged. They are carried from here by lymph currents to the salivary glands, from which they are discharged through the veneno-salivary duct when the Anopheles bites a new victim.

The reader is referred to the diagrammatic sketch (Fig. 23) which illustrates the steps in development.

The Study of the Stained Specimen.—In studying a stained specimen, care should be taken to prevent mistaken diagnosis. A frequent error on the part of students is to identify a platelet superimposed upon a red cell as a plasmodium. This error should not be made if one has sufficient familiarity with the appearance of platelets. Furthermore, careful focusing will show the suspected body and the red cell to be in two different planes. Then the central portion of the red cell may show a markedly increased central pale area. This should not be taken for a parasite. In mounting a peculiar artefact is sometimes seen, giving the appearance of an air-bubble lying on the red cells. This is usually due to insufficient drying of the cover-slip before mounting. Though they do occur, mistakes should not be made on this score because of the refractility of the circular artefacts and their great number. Bits of leukocytic nuclear fragments might also cause confusion, which should be prevented by close scrutiny and adhering to the requirements of a definite and complete morphology before making a diagnosis.

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[1] While the trend of opinion is that the life of the parasite is intracellular, Mary Rowley-Lawson has advanced evidence tending to confirm the view long held by some, that the parasite is extracellular during its whole life cycle. She asserts that it migrates from red corpuscle to red corpuscle, destroying each before it abandons it; that in the brief interval between, the parasite is free in the blood serum; and that it almost immediately attaches itself to another red corpuscle by means of delicate pseudopodia.

Infection with Plasmodium Vivax (*Benign Tertian Malaria*).—Infection with P. vivax is responsible for most instances of malaria seen in temperate and semitropical regions. The period of asexual development consumes forty-eight hours, so that segmentation and accordingly a paroxysm occurs at forty-eight-hour intervals. A daily chill would be possible if the patient were bitten on two occasions, so that sporula-

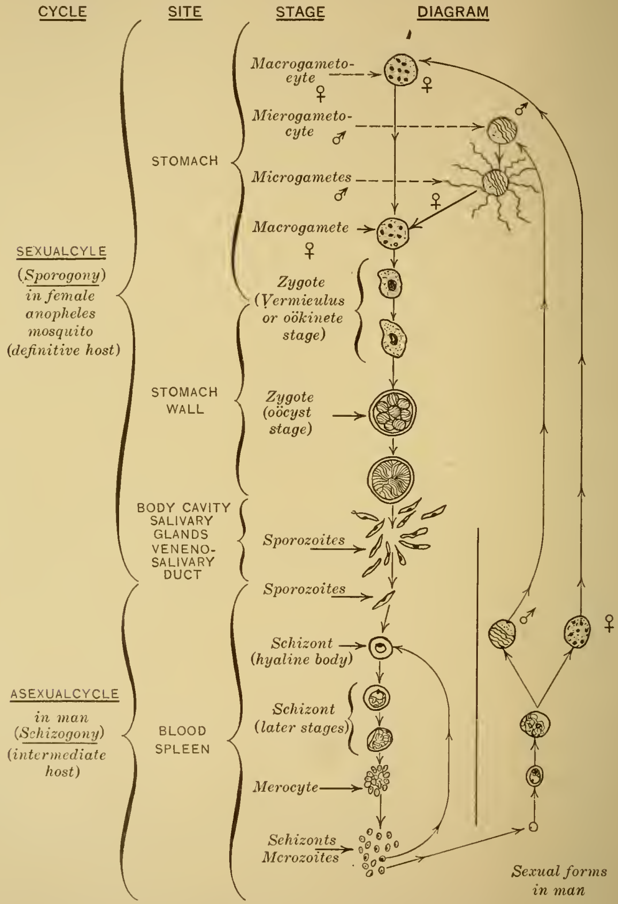

FIG. 23.—Diagrammatic representation of the life-history of the malarial parasite.

tion could occur on alternating days. The blood taken shortly after a chill usually shows numerous young "hyaline bodies," merozoites, each occupying a red cell. These are tiny rings in one red cell. At the same time, the blood may show some older forms, since all the parasites do not develop absolutely simultaneously. As development proceeds, the body assumes an ameboid form or a looped figure, and at twenty-four hours occupies one-half or more of the infected cell, which is swollen to about one and a half times its usual size and is pale and anemic looking. The parasitic body is now termed a schizont. Fine brown pigment is scattered through the body of the parasite.

The parasite continues to increase in size up to the end of the forty-eight-hour period when the pigment clumps at the center, the chromatin divides and from 15 to 20 small circular blue bodies are found, each with a bit of chromatin. This form is sometimes spoken of as a "rosette." After sporulation, there are set free from 15 to 20 sporozoites (hyaline bodies), each of which attacks and enters a new red blood cell.

The gametes resemble the grown asexual forms, except that the ring is thicker while the chromatin is in one mass and the pigment is scattered. This is exactly opposite to the arrangement in the full grown asexual forms. The identification of asexual forms in the P. vivax infection may be difficult for a beginner and is not of especial practical importance.

Infection with Plasmodium Malaria (*Quartan Malaria*).—The period for the completion of the asexual cycle is seventy-two hours, so that the chills occur at seventy-two-hour intervals. With a single quartan infection, there would then be two afebrile days between each day marked by paroxysm. If the patient were infected twice so that the blood contained two sets of parasites, sporulating on different days, there would be two febrile days, one day without chills and fever, again two febrile days, and so on. A number of combinations are possible, such as a quartan and a tertian infection. These possibilities suggest themselves to the reader.

The parasite as seen in the stained preparation offers certain points of difference from P. vivax. The hyaline rings are thicker and the schizont (partly grown form) shows coarser pigment granules which are usually grouped peripherally about the edge of the slightly ameboid, oval parasite. The infected cell is usually somewhat shrunken. The presegmenting form is more symmetrical, the segmentation is into a few sporozoites (8 and 10), and the pigment arranges itself along the lines of division between the merozoites instead of at the center, as with P. vivax.

Infection with Plasmodium Falciparum (*Estivo-autumnal Malaria*).—With estivo-autumnal malaria, the paroxysms may occur every other day. The hyaline bodies are smaller and more delicate in outline. As development proceeds, the asexual forms disappear from the peripheral circulation and one is able to find only a few hyaline bodies and possibly the sexual forms (gametes). When only hyaline bodies are found on repeated examination, one's suspicions should be directed at once to the possibility of an estivo-autumnal infection. The gametes are characteristic of this infection, appearing as ovoid or crescentic bodies. In the case of the crescents, the red cell may be seen, appearing to fill in the space between the two rounded horns of the crescent. This is sometimes found in the "bib." The female gamete (macrogamete) is larger, more granular, does not give off flagella, the cell body stains more deeply, and the pigment surrounds a scanty and compact chromatin mass in the center. The male (microgametocyte) gives off flagella, is more delicate and hyaline, its entire body stains more faintly, the pigment is scattered throughout it, and chromatin is more abundant.

Some workers (notably Craig) hold that there are two varieties of P. falciparum, the cycle with one being twenty-four hours, with the other forty-eight hours.

Differentiation Between Types of Malarial Infection.—Clinically, the temperature curve should be considered, bearing in mind the fact that chills on alternating days may be produced by P. vivax or P. falciparum infection, and also the fact that the patient may be suffering with a multiple infection with one or more species of parasites. The following table summarizes the chief differential points found in examining the stained specimen.

PRINCIPAL DIFFERENTIAL POINTS.

	Plasmodium vivax (benign tertian).	Plasmodium malariæ (quartan).	Plasmodium falciparum (estivo-autumnal).
Appearance of infected red cell	Larger size, lighter pink than normal	Normal size and staining	Distortions, some polychromatophilia and strippling.
Hyaline body (young schizont)	Chromatin mass single	Rings thicker and round	Rings small and very fine; often 2 chromatin dots.
Partly grown schizont	Chromatin in single mass; body with vacuoles or in looplike arrangement	Body more marked, extending as a band across the red cell	Seldom found in circulating blood.
Pigment as seen in mature schizont	Abundant fine pigment distributed throughout parasites	Coarse pigment arranged peripherally	Seldom found in circulating blood.
"Presegmenting form"	Irregular division into fifteen or more merozoites	More regular division into ten or less merozoites	Seldom found in circulating blood.
Sexual forms	Difficult to distinguish in peripheral blood from mature schizont	Difficult to distinguish in peripheral blood from mature schizont	Typical crescentic and ovoid bodies.

Summary of the Blood Changes.—The changes in the blood picture are summed up by Craig as follows: "A marked reduction in the number of red corpuscles, both by parasitic invasion and as a result of poisons elaborated by the plasmodia during their development, as well as changes brought about in the blood-forming glands by the malarial infection; a corresponding reduction in the number of white cells, with, in most cases, a relative increase in the large mononuclear leukocytes; a marked reduction in the hemoglobin, and the presence of black and brownish-yellow pigment, in greater or lesser amount."

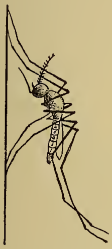

Fig. 24*a*

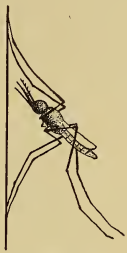

Fig. 24*b*

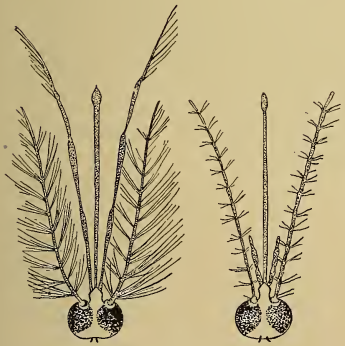

Fig. 25

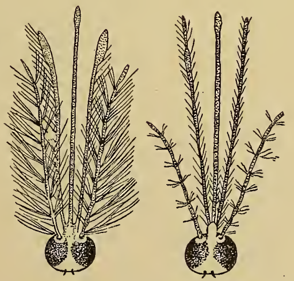

Fig. 26

Comparison of chief characteristics of Culex and Anopheles. Fig. 24*a*, the curved body of Culex when resting is approximately parallel to wall while the straight body of Anopheles. Fig. 24*b*, stands out at an angle of about 45°. In Culex (Fig. 25) the palpæ, of the female are short, while those of the male are longer than the proboscis. In the Anopheles, the proboscis of both sexes are approximately equal in length.

The Malarial Mosquito.—The Anophelinæ may be distinguished from non-malarial mosquitoes by certain characteristic features.

The palpi of female Anophelinæ are as large or larger than the proboscis. The palpi of the Culicidæ are short and stubby. The males of both subfamilies have long, plume-like palpi. With the Anopheles, the proboscis is in the same straight line as the rest of the body, and in resting on a wall, the body projects almost at an angle of about 45°, supported by the first two pairs of legs, the last pair projecting into the air. The Culex, however, rests in a "hunched back" position with the body bent.

Cultivation of the Plasmodium.—The following method has been introduced by Bass and Johns. Blood is obtained by venipuncture under aseptic conditions and placed in a sterile tube, about one inch in diameter, and long enough to go in the centrifuge, containing 0.1 cc. of 50 per cent. glucose solution for each 10 cc. of blood. The blood is defibrinated by stirring with a sterile glass rod. It may be kept in the same tube or transferred to other tubes. In either case, the column of blood must be one to two inches deep. There should be a column of serum at least ½ inch to 1 inch above the cells. The culture tubes should be incubated at 40° C. The parasites develop in the upper layer of precipitated cells. Red cells from this layer may be withdrawn with a sterile capillary pipette for staining and study. Cells from a lower layer contain only dead parasites. If more than one generation is desired, the leukocytes must be removed when the culture is made since they destroy the parasites at the time of segmentation. The freshly drawn blood is placed in a sterile tube which will fit in the centrifuge. The centrifuge should be run at a speed of 800 to 2000 revolutions per minute. The speed should be such that it will force

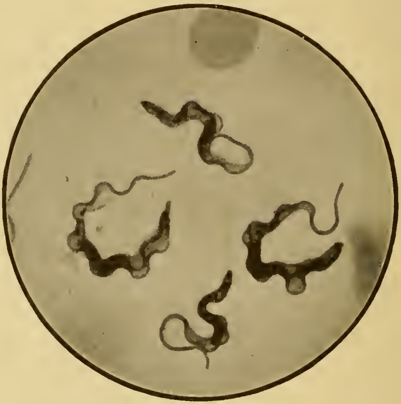

Fig. 27.—Trypanosoma gambiense showing the usual form; some cells in process of division. Magnification 1500 times. (From Novy.)

the red cells to the bottom of the tube and the leukocytes to a layer on top of the red cells. The exact length of time varies with the speed, the length of the centrifuge arms, etc., and must be learned by experiment. When centrifugalization has been carried out properly, the supernatant serum is drawn off and placed in culture tubes. Cells and parasites are now drawn with a pipette from the middle of the cell column and placed at the bottom of the culture tubes underneath the layer of serum. It is advantageous to employ flat bottom tubes.

Trypanosomiasis.—This is a febrile disease, characterized by wasting, edema of the lower eyelids, an erythematous eruption, localized cyanosis, weakness and often lethargy, so-called "sleeping sickness." It is seen in South Africa and is due to infection with Trypanosoma gambiense (T. hominis, T. Castellani). The infection is carried by a species of tsetse fly, Glossina palpalis. A more virulent type of the disease is due to infection with the Trypanosoma rhodesiense, transmitted by Glossina morsitans (Stitt), is seen in South Central Africa. The trypanosomes are blood flagellates, with a fusiform body, an undulating lateral membrane and two chromatin-staining areas. For diagnosis, peripheral blood may be used, preferably by a thick-smear method. Staining can be accomplished by Wright's stain, as for the malaria parasite (Fig. 27). Stitt recommends mixing about 10 or 20 cc. of citrated salt solution, centrifugalizing two or three times, and examining the sediment after the last sedimentation, spreading it upon a slide and staining suitably. In severe cases the organisms may be discovered in the cerebrospinal fluid. Centrifugalization and examination of the sediment is desirable.

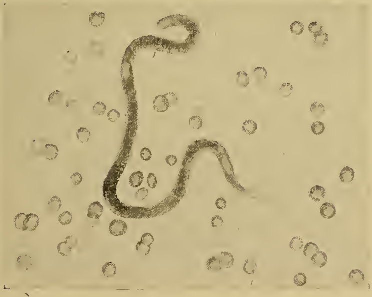

Fig. 28.—Filaria sanguinis. (Simon.)

Filariasis.—There are four principal species of the genus Filaria which produce the disease known as filariasis. They are Filaria bancrofti, Filaria loa, Filaria perstans, and Filaria medinensis. They belong to family filariidæ of the class nematoda. Of these, *Filaria bancrofti* is the most common and it is the cause of more frequent manifestations, such as hematuria, chyluria, elephantiasis, and lymphatic obstruction.

The worm lives in the lymphatics of the trunk and extremities, the male and female being found together. The male is about 2 inches long and the female about 3 inches long. The embryos are found in the circulating blood, showing nocturnal periodicity. They are about 0.2 to 0.4 mm. long, and are sheathed in a gelatinous envelope. When stained, a number of nuclei are seen (Fig. 28). In examining the blood in suspected cases, the specimen should be secured, fixed by flooding with methyl alcohol for two minutes, and then stained with thionin or hematoxylin. It is both unnecessary and inadvisable to stain the red cells.

Numerous reports have been made from time to time of cases of filariasis in the south. Johnston's work is interesting as an indication of the prevalence of the infection. He examined the blood of 400 patients in the wards of a general hospital in Charleston, S. C., in the order of their admission and without regard to the condition for which they were admitted, and found that 19.25 per cent. harbored microfilaria in their blood. In 1919 Edward Francis published a survey of nine other southern cities, and found comparatively few cases. He thinks that Charleston is an endemic focus because of the continued presence of many cases with microfilaria in the blood exposed to constant biting by Culex fatigans.

For detecting the filaria in the blood, Johnston recommends the technic of Rivas and Smith: One cc. of blood is added to 9 cc. of 2 per cent. acetic acid. This is shaken thoroughly and centrifugalized. The red cells are hemolyzed leaving only a small amount of sediment. The sediment is taken up with a capillary pipette and spread upon a slide for examination. While Rivas and Smith found parasites in all infected cases by using this method regardless of the time at which the blood was taken, they found the greatest number of parasites between 8 P.M. and 8 A.M., and between 8 A.M. and 8 P.M. in F. diurna.

Filaria Loa (F. Diurna).—The embryos resemble F. bancrofti closely but have a diurnal periodicity. The adult worms tend to wander about in the subcutaneous tissue, especially that of the orbit. Infection is common in South China, the Pacific Islands, the West Indies, and India. Occasional cases are seen in the United States, most of which are imported. The female worm, which is about 2 mm. in diameter and about 50 to 80 cm. long, is found in the lower extremity. The embryos are evidently swallowed in drinking water and migrate from the stomach to the lower extremity where the female burrows head down to the skin, until the head projects through the skin, an ulcer being formed at this point. The embryos are discharged through this ulcer, especially upon contact with water. Only the female worm was known until recently. The male is quite small and evidently dies and is discharged by the human host.

Relapsing Fever.—Spirochete infection is considered as the cause of the relapsing fevers, S. Duttoni causing the tick fever or relapsing fever found in East and West Africa and S. berbera causing that seen in North Africa and Egypt. The former is transmitted by the bite of an argasine tick, Ornithodorous moubata, and the latter by the bite of infected louse. The spirochetes may be demonstrated in the shed circulating blood (Fig. 29), either by the dark field illuminator or by staining dried films with Wright's stain.

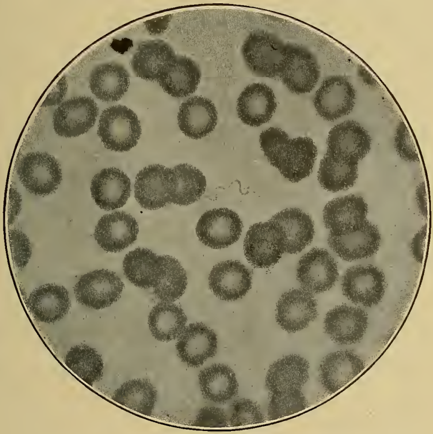

Fig. 29.—Spirochete in the blood. (Meader's case.)

The report by Meader of five cases of relapsing fever in Colorado and the subsequent report of another case by Waring, which had been infected in the same region as had Meader's case, directs our attention to this infection. The carrier in these cases has not been determined.

BACTERIOLOGY OF THE BLOOD.

The technic of blood cultures is not difficult but the identification of the organisms which may be isolated requires training in bacteriological methods. It is unfortunate that blood cultures are not employed more extensively, since positive results are of great clinical value.

Blood should be obtained by venipuncture in sufficient quantity, that is, about 10 to 20 cc. The practice of securing blood from the finger tip or the lobe of the ear is open to objection on two grounds. In the first place, it is impossible to secure a quantity which is necessary for the detection of the organisms when they are few in number. An even more urgent objection is the possibility of contamination.

Apparatus.—A syringe of the Luer type is an ideal instrument, since the form of construction does away with difficulties which arise when washers are used and when pistons are made to fit by wrapping them with some sort of packing. The syringe should be tested before sterilization with water to determine that it draws well and evenly, not with a jerky motion. The best safeguard is to purchase only syringes made by manufacturers of established reputation.

The advantage about a syringe is that it may be used under practically all circumstances. A number of other devices have been suggested. Stitt has proposed one which is described elsewhere (page 124). The writer has modified this somewhat (page 125). With either form of apparatus, the blood is drawn into normal salt solution containing sodium citrate or sodium oxalate. It must be transferred from this to the media into which it is to be inoculated.

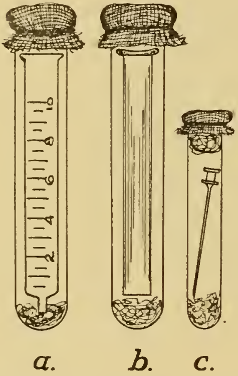

Fig. 30.—Sterilization of syringe (*a* and *b*) and needle (*c*) in test-tubes for blood cultures.

Especial pains should be taken to have a well-sharpened needle. Time spent in putting the needle in condition by sharpening the point and by rubbing off any rust which may have formed on the shaft will be well spent, since the puncture will be made much easier for the operator and much less painful for the patient. It will be found that a short needle will be easier to handle and that the blood will flow more readily if the gauge be moderately broad, about 20 gauge. An irido-platinum needle is desirable, since it does not corrode, retains a sharp point well, and may be passed through the flame just before using without drawing the temper.

The only other equipment required includes a piece of rubber tubing about two feet long for a tourniquet, alcohol lamp, sterile gauze or cotton, alcohol and tincture of iodine for sterilizing the skin, culture medium, and sterile pipette for transferring the mixture of blood and citrated salt solution to the media, if the blood be obtained in this way.

The apparatus must be carefully sterilized. This may be accomplished by autoclaving the syringe and needle or other bleeding apparatus at twenty pounds pressure for twenty minutes. It is convenient to place the barrel of the needle in one large test-tube, the plunger in another, and the needle in a third smaller tube, stoppering all with a well-fitting though not tight cotton stopper (Fig. 30). These may be autoclaved and carried readily. When they are used, the plunger should be taken out by the end of the handle and slipped into the barrel while the latter is still in its tube. The assembled syringe may now be withdrawn, still handling it by the extreme end of the handle, and the tip may be fitted into the butt end of the needle which is in its test-tube, point down. The needle may be picked up in this way without touching it and the apparatus is ready for use.

If more convenient, the apparatus may be boiled for fifteen minutes in 1 per cent. aqueous solution of sodium carbonate, or sterilized for an hour at 150° C. with dry heat. Boiling in ordinary water should not be depended upon for it may not kill spore-bearing organisms.

Venipuncture.—The technic is described on page 126.

The operator need use no precautions regarding the preparation of his hands except those which ordinary cleanliness demand, but should observe bacteriological technic rigidly, touching with his hands nothing that comes in direct contact with the blood. The syringe should be handled as one handles the platinum loop in the bacteriological laboratory, by the distal end only.

Inoculation of Media.—When the syringe is employed, the inoculation of the blood into the culture media must be made at once. Varying quantities, ranging from 1 to 3 cc. or even more may be ejected into flasks of sterile bouillon. The cotton stoppers should be removed and the mouths of the flasks passed through the flame first. After the blood has been added, the end of the cotton stopper which fits into the flask should be burnt in the flame before its insertion into the flask. When plates are made with agar or gelatin, the medium should have been liquified in boiling water and the tubes should have been placed in a tall receptacle containing water at 35° C. to cool the contents to a temperature of 40° C. The blood may be injected into the agar tubes from the syringe just as it was placed in the flasks, flaming the end of each tube carefully before adding the blood. With the tube held in a position as nearly horizontal as possible without letting the contents run out, it should be rolled as soon as the blood has been added to it between the palms of the hand to mix blood and medium thoroughly, the mouth is again flamed, and the contents are poured quickly into a Petri dish. In pouring, the cover should be lifted from the Petri dish as little as possible, to prevent air contamination. After the medium has thoroughly solidified, plates should be inverted, and the two portions should be held together with a strip of adhesive plaster or a broad elastic band. All the inoculated media should be placed in the thermostat at 37° C. at once.

If either Stitt's or the writer's form of apparatus be employed and the blood be taken into citrated or oxalated salt solution, the container may be tightly stoppered with a sterile stopper and taken some distance to the laboratory or even sent by mail. It is preferable, however, to make the cultures into suitable medium as soon as possible. The citrated blood should be transferred with a sterile pipette to the culture medium and should not be poured.

When the cultures have been made, they should be observed for a period of at least a week before being pronounced sterile.

Types of Media.—The types of media to be employed will depend to some extent upon the infection which is suspected. For this reason if for no other one, the laboratory worker should be given enough of a knowledge of the case to provide suitable media.

A good medium for general purposes is meat-infusion agar or meat-infusion broth which has been enriched by the addition of 2 per cent. of glucose. The same medium may be used without glucose, though its addition makes it suitable for a greater variety of organisms, particularly after the addition of human serum.

Anaërobic cultures may be made by placing medium and blood in long tubes and by exhausting the oxygen in the usual way or by using Robinson's meat medium. They should be made when infection by the Bacillus aërogenes capsulatus or other anaërobic organisms is suspected.

When typhoid or paratyphoid fevers are suspected, care should be taken not to have the media too highly acid. The use of an ox-bile medium has been recommended by many workers. This may be prepared by dissolving inspissated ox-bile (Merck's), 30.0 grams and peptone, 2.5 grams, in distilled water, 250 cc., and by autoclaving the solution in tubes. It is not necessary to use this medium, since typhoid bacilli will grow upon ordinary media, though bile has an inhibitory effect upon other contaminating organisms, and for that reason is preferred by those who take blood for cultural purposes from the finger tips or ears. When attempting to demonstrate typhoid group a relatively small amount of blood should be added to the bouillon flask, in order to avoid by the dilution the bactericidal effect of the blood serum.

When one expects to find pneumococci or streptococci, it is well to use medium preferably containing glucose. This may be accomplished by adding to several flasks of the glucose-meat-infusion-broth which was recommended as a more or less universal medium, one or two grams of powdered calcium carbonate. Needless to say, this should be added before sterilization. Litmus milk also makes an excellent medium for the pneumococci. The exact directions for making media suitable for cultivating the pneumococcus are given in the chapter on Sputum (page 362).

In attempting to isolate gonococcus from the circulating blood, it is well to have media which is neutral to phenolphthalein, and it is preferable to have it made from a veal infusion rather than from a beef infusion. Especial care should be taken to maintain the temperature of the incubator at 37° C. Relatively large quantities of blood should be added.

In order to permit growth at different gradients of oxygen tension, Rosenow recommends making shake-cultures in the columns of ascites-dextrose-agar. The blood is taken in the usual way and decalcified by placing it in a sterile container with 2 per cent. sodium citrate in normal salt solution. The proportion should be five volumes of blood to one of the citrated salt solution. A portion of the blood is used for making cultures in flasks and in plates in the usual way. The remainder is transferred by means of a sterile pipette to sterile distilled water, in the proportions of 1 cc. of citrated blood to 10 cc. sterile distilled water. The water should be in round bottom 50 cc. centrifuge tubes. These are centrifugalized and the sediment is removed with a sterile pipette and planted. The treatment with water serves to remove the hemoglobin and serum.

The sediment is planted into long tubes containing a column from 10 to 20 cm. high of dextrose-agar enriched with ascitic fluid. The agar should be 1 per cent. acid to phenolphthalein. The ascitic fluid should have been collected aseptically and should have been sterilized by heating at 60° C. for one hour on three successive days. One part of ascitic fluid is added to nine parts of agar after the agar has been boiled in a water-bath or steamed in an Arnold sterilizer for fifteen minutes to drive off the molecular oxygen. The heated agar should be cooled to 60° before adding the ascitic fluid. The medium is then cooled to 39° C. and the sediment from the laked sterile blood is added with a sterile capillary pipette, inserting the mouth of the pipette to the bottom of the tube containing the medium and forcing out the contents of the pipette as it is withdrawn. The cotton plug is replaced in the tube and the contents are mixed by partially inverting the tube several times. When planting the sediment, care should be taken not to blow any bubbles of air into the medium. Rosenow states that the space between the bottom and top of the tubes represents different gradients of oxygen, since the living cells in the bottom of the tube have oxygen-consuming power and so exhaust the oxygen, while the top remains aërobic.

Colonies in the depth of the ascitic-dextrose-agar are examined by breaking the tube with a glass cutter and the red-hot end of a glass rod, flaming the surface of the tube just before breaking in order to sterilize it. The colony is fished out and suitable subcultures are made, reproducing in the subcultures as nearly as possible the conditions under which growth originally took place.

To give directions for identifying the type of organism falls outside the scope of this work, except for such aid as may be secured in the section on simple bacteriological methods. If a growth appears subcultures should be made upon suitable media. If typhoid fever be suspected, and one finds a freely motile, Gram-negative bacillus, which fails to produce gas with glucose or lactose, the reasonable presumption is that it is the typhoid bacillus, but this must be confirmed by agglutination tests (see pages 107 and 110).

In cases of septicemia due to streptococcus and staphylococcus infections, the percentage of positive results varies according to the site of the process. Probably the highest percentage of positive results is obtained with ulcerative endocarditis. Osteomyelitis frequently gives positive results in blood cultures. It is felt that the prognosis of staphylococcus infections is worse than streptococcus infections.

The pneumococcus can be demonstrated in a definite proportion of cases, but the demonstration of the pneumococcus by blood culture cannot be said to have any reason for preference over the other clinical and laboratory methods which are more readily applicable. It was found by Avery, Chickering, Cole and Dochez that in cases in which a positive culture was obtained, the course of the infections was invariably severe and in many instances terminated fatally.

The gonococcus has been reported in blood cultures.

The list of other organisms reported as having been found is a fairly long one and serves to illustrate the number of infections which may be accompanied by at least a bacteriemia if not a septicemia.

It has been pointed out by Libman, that the question of general infection is a variable one, depending upon the nature of the primary infection. For example, positive blood cultures are rarely obtained in uncomplicated erysipelas, in abscess of the lactating breast, in liver abscess, appendicitis, peritonitis of intestinal origin, pyelitis of pregnancy, thrombosis of the pelvic veins, pyelophlebitis. He has also called attention to the fact that general infection as evidenced by blood culture is very uncommon with mastoiditis or otitis media, whereas positive blood cultures are obtained when the meninges or lateral sinuses are involved. In rheumatic fever the cultures are usually sterile except for the occasional finding of the Streptococcus hemolyticus, which does not seem to be the cause of the disease, since it is only occasionally present and since complement-fixation tests prove uniformly negative.

The information furnished merely by bouillon cultures and the blood-agar plates gives much information regarding the streptococci. (For discussion of the use of blood agar for the study of streptococci, the reader is referred to the comprehensive work of James Howard Brown.)

In giving consideration to the results of blood cultures, possible contamination should be carefully excluded. This should offer no great difficulty. If all the plates and flasks show a growth of the same organism, if the colonies are found deep in the agar and not merely on the surface, and if the organisms are of types ordinarily pathogenic and not such as might be expected from accidental contamination, it is reasonable to suppose that they had their source in the circulating blood. If, however, the colonies are found on one or two plates only, or in one or two flasks only, if they are on the surface of the plates, and if they are of varieties frequently found on the skin or in the air, the chances are strongly in favor of accidental contamination. Hiss and Zinsser's rule is excellent: "to look upon all staphylococcus blood cultures skeptically, discarding Staphylococcus albus as a contamination, and taking, if possible, another corroborative culture when the organism is Staphylococcus aureus."

Results of Blood Cultures.—In giving consideration to typhoid fever and in the paratyphoid infections, blood cultures are of great clinical assistance. Positive results are especially frequent in the first week of the disease, slightly less so in the second, markedly less so in the third. From this it may be seen that the results supplement those obtained by the Widal reaction, so that by using both methods it is possible to make a definite diagnosis on laboratory grounds in practically all cases. Gay states that when repeated blood cultures are made a positive diagnosis may be expected in from 75 to 85 per cent. of all cases of typhoid fever. The following figures, taken from Gay's work, are of interest:

PERCENTAGE OF POSITIVE CULTURES IN RELATION TO TIME OF DISEASE.

	1st week, per cent.	2d week, per cent.	3d week, per cent.	4th week, per cent.	After 4th week, per cent.
Coleman and Buxton . .	89	73	60	38	26
Mann, Rainsford, Warren .	80	62	50	50	
Gay	73	80	53	53	33

SERUM REACTIONS.

Agglutination Tests.—The Widal Reaction.—The fact that infection by certain microörganisms results in the elaboration by the infected host of defensive bodies known as agglutinins, make it possible to examine the blood for these bodies as a diagnostic measure. For example, blood serum from a typhoid patient, even when considerably diluted, contains agglutinins, which will cause clumping or agglutination of typhoid organisms. The tests based on this principle have assumed considerable clinical importance. The one for typhoid fever is called the "Widal" or "Gruber-Widal" reaction. The test is also applied to the diagnosis of the paratyphoid infections, and occasionally in dysentery and in cerebrospinal meningitis.

The same technic is employed for performing the agglutination tests for typhoid and paratyphoid infections, the only difference, of course, being in the organisms which are employed. Either of two methods may be employed, each of which has certain objections and certain advantages. The microscopic method requires only small quantities of blood and it is completed a little more rapidly than the macroscopic method, which requires a greater amount of blood. The latter is the method of choice for scientific work, because of the ease with which accurate dilutions are made for the purpose of ascertaining the agglutination titer of the serum.

Microscopic Method.—Stock cultures of the organisms to be employed for the tests must be at hand. These may be grown on solid media at room temperature and should be of known agglutinability. They should be transplanted once monthly. For performing the test, a subculture should be made either on an agar slant or in bouillon. The agar slant may be allowed to grow about twelve hours, the bouillon cultures about eighteen hours. The writer's personal preference is for the agar slant culture. If the bouillon culture be utilized, a drop may be mixed with a drop of diluted serum. When agar is preferred, 2 or 3 cc. of normal saline solution should be poured into the tube, which is rolled between the palms a number of times until uniform suspension has been made of the organisms. A drop of the suspension may be used for mixing with a drop of diluted serum.

The only apparatus needed in addition to the microscope consists of hanging drop slides, *i. e.*, object slides with a concavity ground out at the center (Fig. 31, *a*); vaseline or immersion oil for encircling the concavity in the slides; cover-slips; a platinum loop; a fine capillary pipette; a Wright's capsule or a small test-tube for collecting the blood; small watch-glasses, open salt dishes or small, flat staining dishes for mixing the serum to make dilutions.

Normal salt solution should be at hand for making dilutions.

Method.—Five or 10 drops of blood are obtained in a Wright's capsule (Fig. 31, *b*). If a blood culture is done on the patient at the time the blood is taken for the Widal reaction, the last half cc. of blood may be ejected into a small test-tube for the agglutination tests. The serum should be allowed to separate from the clot. This will usually take place spontaneously if the capsule is allowed to remain undisturbed for ten or twelve hours. Separation may be accelerated by centrifugalization. If a capsule be employed, the end which is farther from the blood may be sealed in a low flame, taking care not to heat the blood, and after the glass has cooled, the capsule may be placed, sealed end down, in a centrifuge tube the bottom of which has been lightly packed with cotton. When the serum has been separated, the capsule may be filed at or near the center and broken in two. A drop of serum should be placed in a watch-crystal marked A and a second drop into a crystal marked B. The pipette (Fig. 31, *c*) should be cleaned by drawing and ejecting successive changes of water, absolute alcohol, and ether. The residual ether should be thoroughly dried out by suction. It may then be rinsed with normal salt solution. After thorough cleansing, normal saline may be drawn into the pipette. Then nine drops should be placed in dish A and twenty-nine drops in dish B. A now contains a 1:10 dilution and B contains a 1:30 dilution.

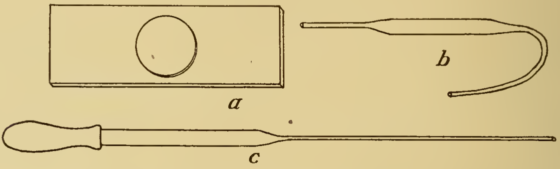

Fig. 31.—Apparatus required for microscopic agglutination tests. *a*, hanging drop slide; *b*, Wright's capsule; *c*, capillary pipette with rubber nipple.

Three hanging drop slides are taken and the edges of the concavity are ringed with vaseline or with immersion oil. On one cover-slip is placed with the platinum loop a drop of the 1 : 10 dilution of serum. To it is added with the platinum loop a drop of the suspension of typhoid bacilli, and the two drops are thoroughly mixed with gentle stirring, taking pains not to spatter any droplets onto the fingers or table top. The cover-slip is quickly inverted and mounted over the concavity of the hanging drop slide with the drop hanging down into the concavity which now becomes a sealed, moist chamber. The dilution is now 1:20. The dilution and time are written on the end of the slide with a grease pencil. The procedure is repeated with a drop of the 1:30 serum dilution, which becomes a 1:60 dilution after the addition of the drop of typhoid suspension. On the third slide should be similarly mounted a cover-slip upon which has been mixed a drop of the typhoid bacillus suspension and a drop of the normal saline solution used as a diluent. This serves as a control on the motility of the bacilli, and should show neither loss of motility nor spontaneous agglutination.

The preparations should be examined at the end of thirty minutes and again at the end of an hour with regard to loss of motility and the clumping of the bacilli into groups of greater or lesser size. In a definitely positive reaction, there should be definite clumping and complete loss of motility; that is, there should be practically no unclumped or motile organisms. The reaction is said to be doubtful when there are only a few clumps and only partial loss of motility. With a negative reaction the appearance of the slides containing serum is similar to that of the control slide, showing no loss of motility and no clumping.

Use of Dry Blood.—The use of dry blood for performing agglutination tests is not good practice, since it is impossible to make accurate dilutions. It is a method, however, which is employed considerably, especially in public health laboratories. It has the obvious advantage of making the sending of blood from a distance much easier. A drop of blood may be obtained from puncture of ear or finger and placed on a glass slide, a bit of aluminum, or even on glazed paper, dried, and sent to the laboratory. Upon its receipt in the laboratory, the dried blood may be rubbed up and dissolved with a little normal salt solution and transferred with the platinum loop to a cover-slip upon which has been placed a drop of the typhoid bacillus suspension. Dissolved blood should be added until the color of the mixture on the cover-slip is a delicate orange. This is assumed to represent a dilution of 1:40.

Precautions Against Infection.—Since living cultures of typhoid bacilli are being handled, great care should be taken to prevent any possibility of infection. The cover-slips on which the drops of serum and bacillary suspension are mixed should be held in cover-slip forceps or mounted on the end of a cork while the mixture is being made and under no circumstances should be held in the fingers. Even the patient's blood should be handled with care, since it may contain typhoid bacilli in the early stages of the diseases. The bacteria are not necessarily dead even after agglutination, therefore the cover-slips should be carefully lifted from the hanging drop slides with a pair of forceps, and the slides, cover-slips, and forceps should be dropped into a solution of phenol or lysol. The table top should be wiped up with one of these solutions.

Macroscopic Method.—Fresh cultures are made on slants of agar, and after twenty-four hours' incubation, the growth is removed and a suspension made in sterile normal saline. This may be heated for one hour at 60°. At the end of this time cultures should be made to be certain that the suspension is sterile. The suspension may be kept in the refrigerator and should be well shaken before use. The suspension must not be too thick. Experience will show the density which the emulsion should have.

A suspension may be made according to the Oxford University method. The bacilli are grown for ten days or two weeks with daily subculturing. At the end of this time a few drops are placed into a larger Erlenmeyer flask, partly filled with veal infusion bouillon. The flask should be sterilized in the autoclave at 115° C. for no more than fifteen minutes and should be incubated before inoculation to make certain of its complete sterility. After inoculation growth is allowed to proceed at 37° C. for twenty-four hours, the flask is well shaken and for each liter of medium is added 1 cc. of commercial (40 per cent.) formalin. The contents are shaken and the flask is placed in the refrigerator at about 2° C. During the same day and on four or five subsequent days the flasks are shaken at intervals and replaced in the refrigerator. At the end of three or four days the bacilli will have been killed. Cultures should be made to determine absolutely the question of sterility. When sterilized, the culture may be made homogeneous by shaking in a mechanical shaking machine, or the larger particles may be removed by filtering through sterile cotton-wool.

The apparatus required will include a small test-tube rack, seven small test-tubes, about 1 x 10 cm., accurately graduated volumetric Mohr pipettes (one with a capacity of 1 cc., graduated in $\frac{1}{100}$ cc.), and an incubator or water-bath maintained at 37° C. Normal saline solution should be at hand.

Seven small test-tubes are placed in the rack and 1 cc. of normal saline is placed in each tube, except the first, which receives 1.8 cc. In the first tube is placed 0.2 cc. of the serum to be tested. The serum remaining in the graduated 1 cc. pipette is discarded, and the serum-salt solution mixture in the first tube is drawn into and ejected out of the pipette to ensure complete mixing. The first tube now contains 2 cc. of a 1 : 10 dilution of serum. After thorough mixing, 1 cc. of this mixture is added to the salt solution in the second tube. The 1:10 mixture remaining in the pipette after 1 cc. has been measured into the second tube is ejected into the first tube and the pipette is then used for thoroughly mixing the contents of the second tube. After this has been accomplished, 1 cc. is transferred to the third tube. The residue is placed in the second tube, the contents of the third tube are thoroughly mixed, and 1 cc. of the mixture is carried to the fourth tube. This process is repeated until the sixth tube receives 1 cc. from the fifth tube. This is mixed as before, and then 1 cc. is discarded. Each tube now contains 1 cc. respectively of the following dilutions, beginning with the first; 1:10, 1:20, 1:40, 1:80, 1:160, 1:320. The seventh tube contains only 1 cc. of normal saline solution. To each tube is added 1 cc. of the bacterial suspension with a graduated volumetric pipette. This results in doubling the dilution in each tube, the dilutions being respectively 1:20, 1:40, 1:80, 1:160, 1:320, 1:640. The seventh tube now contains only normal saline and bacterial suspension, and serves as a control. After setting up the tubes in this manner, the dilutions should be marked on each tube with a grease pencil, the tubes should be shaken and placed in the incubator at 37° C. for two hours. Then they may be allowed to stand at room temperature for six hours or in the ice-box over night, at the end of which time the readings may be taken. The control tube should show only uniform cloudiness. If a slight sediment is present, it should be possible to break it up with gentle shaking. With a positive reaction in the tubes containing diluted serum there will be a marked massing of the bacteria which will adhere to the sides and bottom of the tubes.

If a mixed infection be suspected, the absorption agglutination test of Castellani may be performed; or preferably, agglutination tests according to Dreyer's method, testing the serum of the patient in parallel series of observations against standardized emulsions of the three microörganisms, B. typhosus and B. paratyphosus A and B, and determining the maximum dilution of serum in which agglutination takes place. For the technic of these, reference should be made to standard texts on immunology.

Clinical Significance of the Results.—A positive Widal reaction with typhoid bacillus suspension and with a serum dilution of 1:60 may be taken as evidence of a typhoid infection. While certain other diseases may give partial reactions with lower dilutions, and while even normal serum may clump the bacilli in a 1:10 dilution, it is generally agreed that only typhoid infections will give a positive reaction with a dilution as high as 1:60.

The exact time during the infection at which the patient's serum will first give a positive reaction is variable. While considerable work has been done upon the subject, it is difficult to correlate the results because of the fact that different investigators have used different dilutions, and because no uniform method has been adopted for determination of the day of onset of the disease.

Approximately 80 per cent. of the cases give a positive result in the second week of the infection with a 1:60 dilution, taking as the first day of the disease the day when the patient took to his bed. The percentage of positive results increases somewhat with the progress of the disease and it is stated that if repeated examinations are made frequently enough during the course of the patient's illness, a positive reaction will be secured in about 90 to 95 per cent. of cases. The following figures have been reported as giving the percentage of positive results at different stages of the infection:

PERCENTAGE OF POSITIVE AGGLUTINATION TESTS IN DIFFERENT PERIODS OF TYPHOID FEVER.

	1st week, per cent.	2d week, per cent.	3d week, per cent.	4th week, per cent.	5th week, per cent.	Average, per cent.
Park and Williams	.20	60	80.0	90	...	88.0
Gay	.61	82	87.5	92	100	91.8

Frequently, the most marked agglutination is obtained during convalescence, and a positive reaction may persist for a period of weeks or months. Cases are reported where a positive Widal has been secured years after an attack of the disease. In such instances, the individual should be suspected of being a carrier. It is to be remembered that the inoculation of typhoid vaccines will produce a positive Widal and that this may persist for many months. That a negative reaction cannot be taken as sufficient reason for excluding the presence of typhoid should go without saying.

Another factor which may cause confusion is the group agglutination that occasionally occurs with paratyphoid infections. A typhoid patient may give a partially positive agglutination with typhoid bacilli and one almost equally positive with paratyphoid bacilli. In such cases, the absorption method of Castellani would have to be utilized to clarify the diagnosis.

Isohemagglutinins in Human Serum.—The presence of isohemagglutinins in human sera and the division of individuals into four groups according to agglutination reactions, makes the determination of the group to which donor and recipient belong of much importance when transfusion or skin grafting is contemplated. A donor for transfusion must belong to a group whose cells are not agglutinated by the recipient's serum. Severe and even fatal reactions may follow the injection of improperly selected blood. That the division into groups must be considered in skin grafting also has been shown by Shawan's work. The procedure is referred to as "blood grouping" or "blood matching."

Jansky and Moss has found that all bloods may be divided into four classes. An individual retains his group characteristics throughout his lifetime.

In Group I the serum does not contain any iso-agglutinins, and therefore does not agglutinate the corpuscles of individuals from the same or any other group.

In Group II the serum contains an agglutinin known as agglutinin A. It will cause clumping of the cells of individuals from Groups I and III.

In Group III the serum contains an agglutinin termed agglutinin B. It will agglutinate cells from Groups I and II.

In Group IV the serum contains both agglutinins A and B. It clumps the blood corpuscles from Groups I, II, and III.

The interactions may be represented as follows:

No serum agglutinates its own cells.

Group I serum agglutinates no cells.

Group II serum agglutinates cells from Groups I and III.

Group III serum agglutinates cells from Groups I and II.

Group IV serum agglutinates cells from Groups I, II, and III.

Group I cells are agglutinated by serum from Groups II, III, and IV.

Group II cells are agglutinated by serum from Groups III and IV.

Group III cells are agglutinated by serum from Groups II and IV.

Group IV cells are not agglutinated by any serum.

The group divisions among the population is approximately as follows:

Group I comprises 7 per cent.

Group II comprises 40 per cent.

Group III comprises 10 per cent.

Group IV comprises 43 per cent.

While the grouping of Moss has been accepted generally heretofore, there is an increasing tendency to adopt that of Jansky, because of priority. A serum which would be classified as of Group I according to Moss is placed in Group IV by Jansky. Groups II and III are the same with both classifications.

Lee's Method.—The apparatus required includes a hand lens, hanging drop slides, small test-tubes, and platinum loop.

The reagents needed are normal saline solution and a small amount of sterile serum from Groups II and III. Karsner and Koeckert have shown that this serum may be preserved very simply for a period of months without appreciable loss in its agglutinin titer if placed in small capillary pipettes, hermetically sealed at both ends. When serum is kept in a dried state on glass cover-slips or filter paper, the titer is reduced and non-specific agglutination is seen. Before employing serum for tests, its power of agglutination should be demonstrated. For example, when one part of Group II serum is diluted with nine parts of sterile normal salt solution, it should agglutinate Group III cells.

Blood is obtained from the patient (recipient) and from the proposed donors. In each case the blood is obtained from a puncture of the ear lobe. One drop is allowed to fall into a small test-tube previously charged with 1.0 cc. normal saline solution. The tubes are shaken very gently. One drop of each suspension is mixed with a drop of Group II serum and one drop is mixed with a drop of Group III serum. The platinum loop may be used for measuring the drops and for careful and gentle mixing. The results are to be observed under the low power of the microscope or with a hand lens at the end of five minutes. When agglutination is present, the cells will be seen clumped closely; while separation of the cells through the major portion of the drop is evidence of the absence of agglutination.

The group may be determined with the aid of this chart, a plus sign representing the clumping of corpuscles, a minus sign representing the reverse.

	Group II serum.	Group III serum.
Effect upon cells of Group I	+	+
Effect upon cells of Group II	−	+
Effect upon cells of Group III	+	−
Effect upon cells of Group IV	−	−

For example, if clumping occurs with both drops of cell emulsion from a given blood, the individual who furnished the cells belongs to Group I; if clumping occurs with neither drop, the individual belongs to Group IV; if clumping is seen with the drop mixed with Group II serum and not with the drop which was mixed with Group III serum, the individual belongs to Group III, and conversely, if the agglutination occurs with Group III serum and not with Group II serum, the individual belongs to Group II. In performing tests, it is advisable to set up a control with a known normal blood.

If *Jansky's classification* be followed, the following table illustrates the effect of sera of Groups II and III upon the cells of the four groups.

	Group II serum.	Group III serum.
Effect upon cells of Group I	−	−
Effect upon cells of Group II	−	+
Effect upon cells of Group III	+	−
Effect upon cells of Group IV	+	+

Application of Results.—In transfusions or skin grafting it is ideal to have recipient and donor belong to the same group. This is not always possible, however, and a donor may be used whose cells are not agglutinated by the recipient's serum.

Rous and Turner's Method.—This method has the advantage that known sera are unnecessary since the blood of the recipient is tested against that of the proposed donors. Rinse the 1:10 hemocytometer pipette with 10 per cent. aqueous solution of sodium citrate, and draw citrate solution to the 1 mark. Fill pipette rapidly with blood from puncture of patient's ear or finger, and expel mixture into a small, narrow test-tube. Repeat operation with each of the prospective donors. If several donors are to be tested, 2 or 3 pipettefuls of blood should be taken from the patient.

The mixing of the patient's blood with that of the donor's is done in Wright's pipettes (Fig. 31 *C*), drawing blood to the mark, followed by a small amount of air, and then another portion of blood, repeating the operation until the desired dilution has been obtained. With the blood of each donor, two mixtures are made, one containing 9 parts of patient's blood to 1 of the donor's, and one containing equal parts. The dilutions are ejected on a microscopic slide and drawn well up into the pipette to ensure mixing. After fifteen minutes the sealed end is broken and a small drop of the contents expelled on a slide, superimposing a drop of salt solution, and covering with a cover-slip. Readings should be made with the microscope, noting the presence or absence of agglutinated clumps. When present, these are usually of fairly uniform size in a given preparation and are sometimes large enough to be macroscopically visible.

If clumping be present in the 1 + 9 mixture and to a less degree or not at all in the 1+1 mixture, the patient's blood agglutinates that of the donor and may hemolyze it, and the proposed donor cannot be used for transfusion. Clumping with 1+1 mixture and none with the 1 + 9 mixture indicates that the prospective donor's blood agglutinates the patient's cells and the advisability of transfusion is doubtful. When there is no clumping in either mixture, the assumption is justifiable that the two bloods do not hemolyze or agglutinate each other and that transfusion is safe.

Precipitin Reactions for Human Blood.—Animals which have received injections of a foreign protein (antigen) develop among other substances an antibody for this antigen which is termed precipitin. If serum containing the precipitin be mixed with protein of the sort used for antigen, a precipitate will be formed. This reaction has been taken advantage of in identifying the source of blood and other stains and has been found especially useful for medico-legal purposes. The reactions are not absolutely specific, since an antiserum obtained by injecting human serum into a rabbit will give a precipitation when mixed with cognate species of primates, especially the higher groups of apes, although the result will not be obtained with as high dilution as with human serum.

Blood stains upon cloth, metallic bodies, glass and other objects may be subjected to the test. Stains of great age have given satisfactory tests. The dried blood may be scraped from a hard surface and dissolved with normal salt solution. If the stain be on a piece of fabric, a portion should be cut out, carefully teased out with teasing needles or cut into fine bits with a pair of clean scissors, handling the fabric only with forceps to avoid the use of the hands, and the bits placed into a thoroughly clean test-tube. A small quantity of 0.85 per cent. sodium chloride solution should be added, and the bits of fabric should be allowed to soak for a period of several hours. The process may be allowed to proceed in the incubator at a temperature of 37° C. for the first few hours and then allowed to continue at room temperature as long as twenty-four hours, if necessary. After extraction, the solution may be filtered through fine filter paper, or even through a Berkefeld filter if necessary, to render it entirely clear. If the solution be not neutral to litmus, it should be made so by the addition of a dilute solution of either sodium bicarbonate or tartaric acid, as the case may be.

The extract must be diluted with normal saline solution to the proper strength, approximately 1:1000. Solutions of the proper strength will show a slight foam when shaken, and they will give only a slight opalescence when the albumin test is done by heating a little in a tube and adding a drop of 25 per cent. nitric acid. The latter test also serves to show that the stain was made by a protein-containing substance.

Before performing the precipitin test, it will be well to ascertain that the suspected stain is really blood by applying Teichmann's hemin crystal test. The benzidine test may be done also, but it is not regarded as satisfactory for forensic purposes.

Preparation of the Antiserum.—Several rabbits or birds should be immunized, since it is difficult to secure an antibody of sufficiently high titer. Large animals should be used. Three injections may be given intravenously at daily intervals, giving 5 cc., 10 cc., and then 15 cc. of sterile human serum. The serum may be titrated ten or twelve days later, and the animal bled after fasting, if precipitation be given by a 1:10,000 dilution. Another method is to give intravenous injections at intervals of five days, giving first 10 cc. then 8 cc., and next 5 cc. Titration of serum is done a few days after the last dose, and the animal is bled if it gives a high titer. If not, further injections may be given, although the mortality is high after the third injection. The animals may be immunized with intraperitoneal injections, but larger doses will have to be employed. The injections may be given and the blood secured for preliminary titration in the manner described elsewhere (pages 131 and 133).

When the results of the titer are satisfactory, the animal may be bled (see page 135) and the serum separated and stored in 1 cc. lots in hermetically sealed glass ampoules, under sterile precautions. The serum should be absolutely clear. If not, it should be filtered through a sterile Berkefeld filter before sealing for storage.

Titration of the Antiserum.—The immune serum must be of such potency that a prompt precipitation will be produced when 0.1 cc. of it is placed in a test-tube and 1 cc. of human serum is floated over it with a pipette. This test should be carried out as one carries out the Heller's test for albumin. A fine white ring should form at the line of junction of the two fluids.

Method of Performing the Test.—For the reaction slender test-tubes should be employed, preferably about 3 mm. in diameter and 10 cm. long. They should be carefully cleaned and should be free from any chemical impurity, although bacteriological sterility is not essential. Six tubes should be set up in a rack and suitably numbered. Controls must be used to demonstrate the fact that the antiserum will produce precipitation in the presence of human serum, that the unknown will not produce a precipitate with normal rabbit serum, and that neither the antiserum nor the unknown will form a precipitate with the salt solution employed in the test. Furthermore, when the stain has been extracted from cloth or paper a bit of the same material without stain should be extracted with normal saline solution and treated with the antiserum, since certain chemicals used in dyeing might give a precipitate. The following protocol serves as a guide to setting up the test and its necessary controls:

Tube 1. 0.1 cc. antiserum; 1.0 cc. unknown solution to be tested.
Tube 2. 0.1 cc. antiserum; 1.0 cc. known human serum, 1:100 dilution.
Tube 3. 0.1 cc. normal rabbit serum; 1.0 cc. unknown solution to be tested.
Tube 4. 0.1 cc. normal saline solution; 1.0 cc. unknown solution to be tested.
Tube 5. 0.1 cc. antiserum; 1.0 cc. normal saline solution.
Tube 6. 0.1 cc. antiserum; 1.0 cc. normal saline extract of same fabric (when stain is on fabric).

The readings for medico-legal work should be taken in twenty minutes. The results in the first tube will depend upon the presence or absence of human blood in the suspected stain. A precipitate will appear if blood were the cause of the stain and no precipitate should be seen if blood were not present. The second tube is a control on the potency of the antiserum and should show a precipitate. No precipitate should appear in tubes 3, 4, 5, or 6.

Sources of Error.—The substance on which the stain is found may interfere with the test. With stains on leather the conditions are unfavorable, since tannin or other substances used in tanning may cause precipitation of serum. Stains on earth are unsatisfactory. The lime which is usually present will have to be removed from the solution by precipitation with a current of carbon dioxide and filtration. It is to be remembered that the reaction will be given by higher species of apes, though if the proper dilutions are made possibility of confusion is reduced. Care should be taken definitely to establish the fact that the stain is due to blood, since any body secretion containing serum will give the reaction.

Precipitin Reactions for the Detection of Other Proteins.—The same principle may be applied in establishing the species from which unknown proteins come. The reaction is utilized, for example, in determining whether a given sample of meat is admixtures of beef with pork instead of being pure beef or whether so-called sausage contains game or not. The antisera would be obtained by the injection into rabbit's blood from the species whose sera are to be tested. The technic would be the same as has been outlined. If a specimen of meat were to be examined, care should be taken to secure the specimen from the central portion, to cut it into small pieces with a sterile knife, and to mince it thoroughly with a perfectly clean chopping knife or meat grinder. An infusion should be made with normal saline solution for a period of about six hours at room temperature. The concentration of the extract should be adjusted so that it will be sufficient to produce a foam when shaken, but not strong enough to produce more than an opalescence when tested with heat and 25 per cent. nitric acid, as directed in the preceding section. If the substance is fatty, the fat should be extracted by letting the specimen stand in an Erlenmeyer flask over night covered with a mixture of equal parts of chloroform and ether. In the morning the ether-chloroform mixture is poured off, the meat is washed with normal saline, and extraction with normal saline is carried out as previously directed.

The titration of the antiserum and the actual setting up of the test should be carried out as directed in the preceding section.

Complement-fixation Reactions.—In regard to the group of complement-fixation reactions, certain things may be said in common. They are all time-consuming and no method has been devised for materially reducing the required time and labor without a simultaneous sacrifice of reliability in the result; the hemolytic system requires painstaking attention to quantitative methods and a correspondingly accurate technic; the greater the number of specimens examined at a time with one set of reagents adjusted each to the other, the more dependable will be the results. The biological reagents which are required are not prepared without considerable labor and do not keep indefinitely. Unfortunately it has not been possible to simplify the technic so that any of the dependable complement-fixation reactions may be done unless time, adequate equipment, control sera, and training be available. The dependability of the results will vary proportionately with the experience of the operator, his attention to minute and painstaking detail, the accuracy of his technic, the number of reactions which he is doing.[1]

Principle Underlying Complement-fixation Reactions.—Before proceeding with diagnostic serologic work, a preliminary knowledge of the fundamental principles of hemolysis and immunology is essential. The student should have a laboratory course such as outlined in the excellent manual of Zinsser, Hopkins and Ottenberg, "Laboratory Course in Serum Study," supplemented by study of reference texts, such as Kolmer's "Infection, Immunity and Specific Therapy," or Zinsser's "Infection and Resistance."

The underlying principle is the fixation of the complement by the antigen through an amboceptor. The complement is present in all sera. The antigen is the inciting cause of the disease, the amboceptor is a specific defensive substance (antibody) elaborated by the body in response to invasion of the antigen. In the Wassermann reaction, however, the antigens employed are *not* prepared from the exciting cause of syphilitic infection (Treponema pallidum) but were at first extracts of organs presumed to be rich in treponemata, of the liver of a syphilitic fetus, etc. It was found that extracts of normal organs, fractions of these extracts, or extracts reinforced by cholesterol gave excellent and apparently specific results when checked with the clinical diagnosis. Antigens prepared in this way give a much higher percentage of positive results in known cases of syphilis than do antigens made from pure cultures of treponemata. Therefore, the Wassermann reaction as performed at present does not employ a specific antigen and rests on empiric grounds, which clinical experience has shown to be quite secure. The complement-fixation reaction for gonorrhea and tuberculosis employ specific antigens and rest theoretically on a secure basis, but the practical results in tuberculosis are vastly less satisfactory.

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[1] The list of apparatus needed and the directions for cleaning glassware are given in the Appendix.

In the actual performance of any of the tests of this group, the reaction produces no *visible* change though we may place together antigen, complement and the patient's serum containing antibody and though fixation of the complement may take place. It is necessary then to employ some means of determining whether or not fixation occurred. For this purpose the so-called "indicator" or "hemolytic" system are used. After time has been allowed for the fixation of the complement, there is added to the tubes, red blood cells of a given species and hemolytic amboceptor. The latter is serum from a second species which has been immunized to the cells of the first. A number of hemolytic systems are used but in this manual we employ sheep cells and serum of rabbits immunized against sheep cells. In the "indicator system" the sheep cells are the antigen, and the rabbit serum contains the antibody or amboceptor. The complement is obtained from that originally placed in the tube, if any be still available. After treatment at suitable temperature, the "indicator system" shows either hemolysis, partial hemolysis, or no hemolysis, results which are clearly visible. The nature of the result will depend upon the availability of complement, and this in turn upon whether or not it has been fixed by the syphilitic antigen in the presence of a syphilitic antibody. For example, complete hemolysis (solution of the red cells) means that complement was still available, that this had not been fixed by the antigen of the first system and therefore that there was no specific antibody in the patient's serum. On the other hand, lack of hemolysis (no solution of the red cells) means that no complement was available, that it was fixed by the antigen of the first system, and that there was a specific antibody present in the patient's serum to effect this fixation. While the reaction may seem simple enough in theory, several factors have to be considered in its practical application. The quantitative relationship of the hemolytic or indicator system must be nicely balanced; the amount of the antigen employed must be sufficient to fix the complement in the presence of syphilitic antibody but not great enough to give non-specific fixation; the human serum must be freed from the complement which it contains to avoid adding additional complement, and also from anticomplementary substances; and sera must be devoid of marked bacterial contamination since this may give rise to a falsely positive result. These points, which must be safeguarded technically, will be discussed in detail in the description of the method.

THE WASSERMANN REACTION.

General Considerations and Comparison of Methods.—*Indicator System.* —The original technic of Wassermann, Neisser and Bruck has many modifications, and one who begins this work is forced to make choice between them. Various indicator or hemolytic systems have been suggested. The two which have the most popularity are the one utilizing sheep corpuscles (as used in the original technic) and the one which employs human corpuscles and the hemolytic serum of a rabbit immunized against human corpuscles (as suggested by Noguchi for his modification). The method here described provides for the use of the first-named system. It is preferred because of the general availability of sheep's blood and the ease with which hemolytic amboceptor is developed in rabbits. An antihuman hemolytic amboceptor of corresponding strength is elaborated with much greater difficulty, and the mortality among rabbits subjected to immunization against human cells is high. The system employing sheep cells has one drawback, the presence in many human sera of a native hemolytic amboceptor for sheep corpuscles. In the method given here we attempt to obviate this objection, at least partially, by adding no rabbit serum hemolytic amboceptor to serum which contains native antisheep amboceptor, and by accurate titration of complement which, as Ecker and Sasano have shown, reduces the possibility of error even if excess of amboceptor be present. (In the writer's opinion the best method for performing the test with human cells is the method described by Craig in "The Wassermann Test," C. V. Mosby Co., St. Louis, 1918.)

Inactivation of Human Serum.—The human serum to be tested is inactivated, that is, subjected to a temperature of 56° C. for thirty minutes to destroy the thermolabile complement and certain anticomplementary substances. Omission of this step has been urged, especially by Noguchi, chiefly on the ground that a portion of the specific antibody may be destroyed. However, most workers think that it is better to run the risk of destroying some of the specific antibody rather than the greater one of adding unknown quantities of complement and of introducing anticomplementary substances. This is particularly necessary where cholesterolized antigens are employed.

Antigens.—As has been stated, the antigens employed in the Wassermann reaction are really not specific. A number of types are now in general use, including pure alcoholic extracts of heart (human, guinea-pig, or beef), the same alcoholic extracts reinforced by the addition of cholesterin, or the lipoid fraction of these alcoholic extracts as recommended by Noguchi. The author's personal preference is for the cholesterol-reinforced alcoholic extract antigens, because of availability, delicacy, uniformity and the ease with which they may be prepared. Considerable controversy has been waged about the reliability of various types of antigen. This will be discussed in a consideration of the interpretation of results.

Temperature for Fixation of Complement.—The temperature which is used to accelerate the fixation of the complement by the antigen and specific antibody is also a point on which workers vary. The temperature originally employed was 37° C., for one hour. This was shortened by some to thirty minutes when treatment by immersion in water-bath supplanted treatment by dry heat. Later fixation in the ice-box for a period of from four to ten hours was advocated.

From the researches of several investigators, it would appear that cold fixation (*i. e.*, permitting the fixation of the complement by antigen in the presence of the syphilitic serum at 8 to 12° C. from four to ten hours) gives a much higher percentage of results than fixation for one hour at 37° C. The explanation has been advanced that more complete fixation of complement takes place during the longer time-interval, and that exposure to ice-box temperature for this period of time is possible while exposure to body temperature would result in marked deterioration of complement. Smith and MacNeal have shown that cold fixation enhances the action of cholesterolized and plain alcoholic extract antigens more than it does that of the acetone-insoluble antigens. Ottenberg found discrepancies between the two methods in 15 of 120 cases in which parallel sets of reactions were performed, the greater number of positive results being obtained by the ice-box method. Of the 15 cases, 7 were clearly syphilitic, 2 were treated syphilitics, and 4 were probably syphilitic.

Quantities Employed.—The quantities prescribed in the original technic were much larger than are used by most workers today, the total bulk of the contents of a reaction tube being 5 cc., using 1 cc. of a $\frac{1}{10}$ dilution of complement. This was extremely wasteful of reagents, and the amount of the reagents has been reduced to fractions of these quantities in practically all laboratories. The exact bulk is immaterial as long as it is large enough to permit accurate measurement. Any method, however, which does not demand the accurate volumetric measurement of all reagents, including the serum to be tested, is to be severely condemned and the writer does not feel that the use of capillary pipettes for measurement fulfils the fundamental requirement of accuracy. There is a great variation in the bore of capillary pipettes as drawn by the average worker, so that the size of the drop delivered varies greatly and a tremendous error is introduced into a reaction where quantitative adjustment of the reagents to each other is absolutely essential.

Modifications.—Many modifications of the Wassermann reaction have been proposed. As may be seen, there are a number of variable factors, *i. e.*, the balance of the hemolytic system, the number of units of amboceptor and complement which are employed, the type of antigen, the strength of the blood-cell suspension, and the temperature at which fixation of the complement is allowed to proceed.

Noguchi attempted to simplify the technic so that the reagents could be prepared in a central laboratory, and the test might be performed by the practitioner with slight laboratory facilities. He substituted a human blood-cell system for the sheep blood-cell system of the original Wassermann technic, and proposed a scheme for the preservation of the amboceptor, complement and antigen in the dried form on paper. An antigen made up of acetone insoluble lipoids was devised, and the use of fresh human serum was recommended. The measurement of the reagents in the liquid form by the use of capillary pipettes was advocated. Certain objections to this method may be made. The preservation of antigen and complement on paper has not been found practical on account of rapid deterioration. It is much more difficult to secure an active antihuman than an antisheep amboceptor of high titer. The use of capillary pipettes as a means of accurate quantitative measurement is open to the criticism which has been made. Fresh human serum contains an unknown amount of complement, introducing a variable factor into the reaction.

Craig modified the original technic by substituting an antihuman system for an antisheep system, making no other marked changes. His method is carefully worked out and should commend itself to one who wishes to employ the antihuman system.

Bauer proposed employing the native antisheep amboceptor present in human sera. The presence of antisheep amboceptor, however, is too variable to permit one to rely upon it solely for carrying out the reaction.

The modification of Hecht-Weinberg was based upon utilizing the native complement in human sera. Both of these are extremely variable quantities, but Gradwohl has devised a method for determining the hemolytic strength of a given serum by titration, and has modified the Hecht-Weinberg. Even though this reaction is delicate, Kolmer has stated that it is open to the same error that may occur whenever a crude alcoholic organic extract antigen is used with an active serum, *i. e.*, the appearance of false positive reactions.

Securing Specimens of Human Blood.—It is preferable to secure this by venipuncture. To obtain enough from the finger or the ear for examination by this technic is difficult, though it may be done. Specimens obtained from the finger or the ear often show a certain amount of hemolysis. Obtaining blood in quantity from the ear lobe will be facilitated by making two punctures about 5 mm. apart on the lower portion of the ear lobe with a *well sharpened* triangular lancet such as the one recommended on page 18 (Fig. 2, *a*). The line of puncture should be transverse to the margin of the ear lobe and the lancet should be pushed up into the tissues of the lobe to the depth of 1 mm. Usually a small vessel will be cut by this technic and the blood flows fairly free into the test-tube.

The method of choice is very distinctly the puncture of an arm vein, a procedure which is simplicity itself, under ordinary conditions and should not cause the patient any inconvenience or more than trivial pain *if properly performed.* It is absolutely essential that the needle be sharp. A fine oil stone and a bit of emery paper should be part of the regular equipment. Rust may be removed from the shaft of the needle with the emery paper. The point should be carefully perfected by sharpening on the oil stone. Many needles come from the makers improperly ground so that behind the point is a high shoulder, as shown in Fig. 33. When this is found it is well to grind down the "hump"

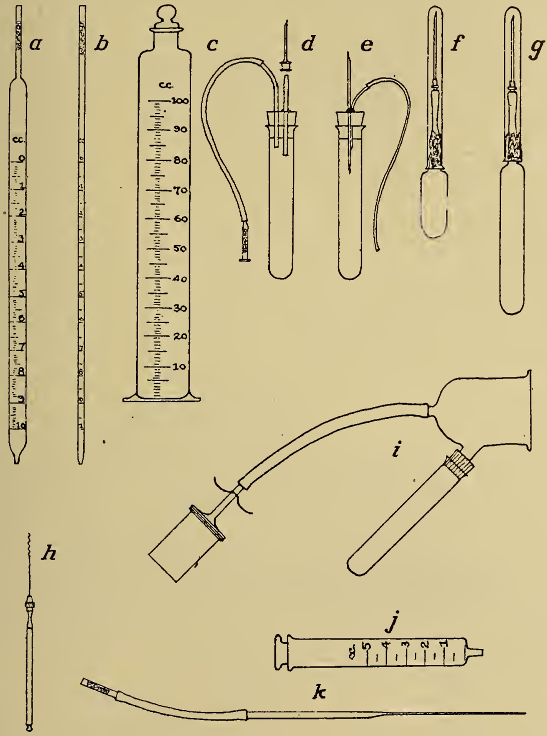

Fig. 32.—Apparatus for immunology. *a*, Mohr pipette, 5 and 10 cc. capacities, graduated in 0.1 and 0.5 cc. respectively; *b*, 1 cc. pipettes, graduated in 0.1 cc. and 0.01 cc.; *c*, glass-stoppered graduated mixing cylinders; *d*, author's device for obtaining blood; *e*, MacRae needle; *f*, Keidel tube; *g*, Keidel tube as modified to fit centrifuge shield; *h*, dental brooch for removing cotton plugs from pipettes; *i*, Blackfan apparatus for securing blood from children; *j*, Luer syringe; *k*, capillary pipette with mouth-piece and rubber connection.

on a carborundum stone until a proper point is secured. The care of the needle is also of great importance. It should be washed out with water immediately after use to remove the blood from its lumen, it should be boiled and dried by running through alcohol followed by ether, unless it is to be used again the same day.

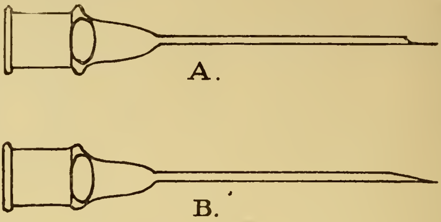

Fig. 33.—*A*, needle-point improperly sharpened; *B*, needle-point properly sharpened.

Several forms of apparatus are available. A bare needle may be used. It comes with the outfits furnished by many of the large public laboratories. It will be found especially bothersome with small veins. It may be inserted with the hand or by gripping it with a stout pair of artery forceps (Fig. 34).

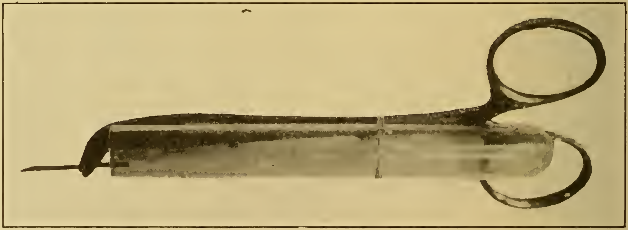

Fig. 34.—Thompson's apparatus for collecting blood.

Partial vacuum is often desirable. The *MacRae needle* has the advantages of dependability and simplicity and a small number of parts to get out of order (Fig. 32, *e*). Here vacuum is obtained by removing the air from the tube by suction. A glass mouth-piece stuffed with cotton may be attached to the rubber tubing to obviate any possibility of drawing the patient's blood into the operator's mouth, but this accident could not occur if rubber tubing is kept above the needle in the position shown in the sketch.

Stitt has devised a piece of apparatus employing the same principle which can be improvised readily in the laboratory at trifling expense from glass and rubber tubing, a rubber stopper and an ordinary needle.

The writer's device, in which an attempt is made to combine the best features of the MacRae and Stitt needles, is more convenient than Stitt's since it may be handled with one hand, and has the advantage over the MacRae needle that there are no metal parts to rust except the needle proper. The size of the needle may be selected for the particular case (Fig. 32, *d* and 37).[1] A solid glass syringe of the Luer type (Fig. 32, *j*) with a slip-joint end to fit the needle, may be used. A syringe which necessitates a washer in the needle is an abomination. Cheap glass syringes prove to be an aggravation, since poor workmanship may lead to leakage and insufficient vacuum.

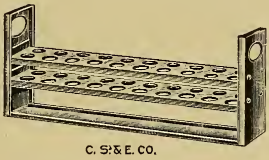

Fig. 35.—Rack.

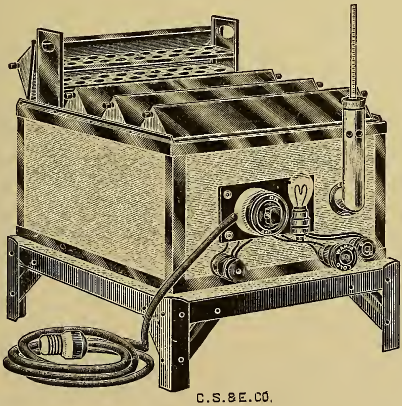

Fig. 36.—Water-bath.

The *Keidel tube* is convenient. This consists of a sealed glass bulb from which the air has been exhausted, to which is attached, by means of rubber tubing, a needle fitted with a stylet. The needle and tubing are covered with a small test-tube (Fig. 32, *f*). When one desires to obtain a specimen of blood, the test-tube covering the needle is removed, the stylet withdrawn, and the needle inserted through the sterilized skin into the vein. With a pair of hemostatic forceps, the glass tip of the vacuum bulb is crushed by pressure on the glass connection and blood should rush into the bulb. When filled it is sealed by replacing the stylet and covering the needle with the test-tube.

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[1] This is manufactured by the Will Corporation, Rochester, N. Y.

The Keidel tube is especially convenient for those who desire to take a specimen of blood and send it to a laboratory. In the laboratory, however, they are nuisances, because it is difficult to remove the blood from them through the narrow neck, and centrifugalization to separate serum from clot before removal would be more or less out of the question since the size of the tube is not adapted to the centrifuge shields. The writer has attempted to obviate this difficulty by having tubes made of thick, tough glass and of the length and diameter to fit the Cornell centrifuge shield (Fig. 32, *g*). When they are received in the laboratory, the top may be removed and the tube put in the centrifuge to separate the serum. These tubes are made by the Steele Glass Co., Philadelphia, Pa. The chief objection to the Keidel tube is the cost, since they can be used only once.

Technic of Venipuncture.—With any form of apparatus, the general procedure is the same. The patient is seated in a chair in such position that a good light falls on the arm. If the patient be in bed and the MacRae or the writer's needle be used, the arm should be so arranged that it will hang down over the edge of the bed. The sleeve is rolled up above the elbow and a piece of rubber tubing drawn tight around the arm above the elbow (Fig. 37). The tourniquet should not be tied, but should be drawn taut, and one end caught underneath as shown in the sketch so that the operator may readily release the tourniquet with one hand by pulling on this end. Experience will show that median cephalic and the median basilic veins exist only in text-books on anatomy and in rare individuals. The skin over the vein is cleansed, preferably by simply applying with a swab a mixture of equal parts of tincture of iodine and 95 per cent. alcohol. This should be allowed to dry. Distention of the vein may be accelerated by directing the patient to alternately clench and open the fist while the tourniquet is in position. When maximum distention has been secured, the needle is inserted through the skin over the vein in a direction parallel to its course and toward the patient's shoulder. Directing its insertion toward the hand is unnecessary and usually inconvenient (Fig. 37). A novice will find it easier to make a quick thrust to carry the point of the needle under the skin and a second one to push it into the lumen of the vein. If suction is employed, it may be used now; should blood fail to appear promptly the point of the needle should be withdrawn slightly, though not from underneath the skin, and reinserted until the wall of the vein has been penetrated. Care must be taken not to go through both anterior and posterior walls of the vein. When the required amount of blood has been obtained, the tucked-under end of the rubber-tubing tourniquet may be pulled, thereby loosening it, the needle may be drawn out and a piece of gauze or cotton placed over the puncture. The patient is directed to make pressure on the cotton with his free hand for a few moments to arrest the bleeding and to prevent extravasation of blood from the vein into the tissues under the skin. Ordinarily the only dressing required is a drop of collodion.

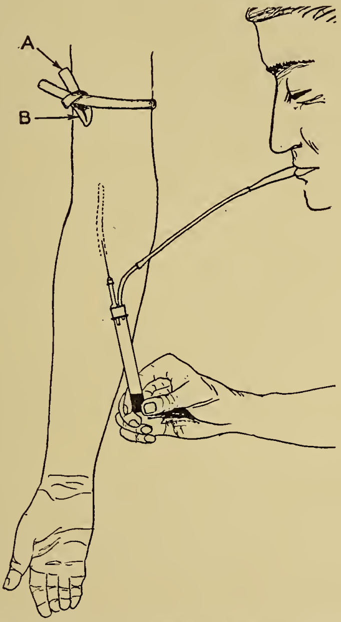


Fig. 37.—Withdrawing blood with author's apparatus. Tourniquet is not knotted, but loop *B* is tucked under. Tourniquet may be detached by pulling on end *A*.

Collection of Blood in Children.—The collection of blood in infants and children may offer some difficulties. (In such cases, the Noguchi reaction has the advantage of employing small amounts of all reagents, patient's serum included.) In infants, the cupping device of Blackfan is helpful (Fig. 32, *i*). The skin of the back below the scapula is scrubbed with alcohol, and is scarified with a sharp scalpel or preferably, with a spring scarifier, previously sterilized by prolonged immersion in alcohol and then dried. The baby is held by an assistant, the cupping device is placed over the scarified area and a vacuum is created by the pump. Hyperemia of the cupped area results and blood flows fairly free. The attached test-tube should be held so that it is dependent in order that the blood may run into it. Usually 2 to 4 cc.

may be obtained without especial difficulty. Ordinarily in older children, from two to four years of age and over, a satisfactory arm vein may be found, but care should be taken to use a sharp needle of small bore and to have the child securely held.

Whenever congenital syphilis is suspected, the blood should be obtained from the infant at the time of delivery by bleeding from the child's end of the umbilical cord before it is tied. This should be a routine measure in the obstetrical wards and hospitals.

Precautions.—Certain precautions are necessary in obtaining specimens. Enquiry should be made as to the recent use of alcohol, since the work of Craig and Nichols showed that the ingestion of beer and whisky in considerable amounts has converted a strongly positive result into a negative one when tests were made within twenty-four hours after taking the alcohol. Bacterial contamination of the blood serum must be guarded against, for Craig found that growth in the serum of certain common bacteria such as staphylococci and streptococci may produce thermostabile anticomplementary substances which would seriously interfere with the reaction. Sera which are heavily charged with bile are often anticomplementary. It is also asserted that blood taken after ether or chloroform anesthesia occasionally shows a false positive result. (Kolmer.)

The specimen tube should be *labelled at once* to prevent possible confusion, promptly *stoppered with a clean cork*, and placed in an upright position to clot. Stoppering with cotton or gauze is inadvisable since either material would absorb the serum should the tube be tipped by accident.

When the specimen is to be examined on the same day, no especial care is necessary regarding its preservation. If it is to be examined on the following day, the serum may be separated when convenient and the specimen kept in the refrigerator over night. When the specimen is shipped to a distant city, the tube should be carefully packed in a stout container, taking care to press down the cork securely. When whole blood is shipped, it should be secured and mailed to reach the laboratory at the time when the laboratory does its Wassermann reactions in order to prevent decomposition. Absolute asepsis is advisable, since Craig has shown that contamination is apt to give false results or at least interfere with the reaction through anticomplementary substances. A wise precaution is to send separated and inactivated serum with a notation as to the nature and date of the procedure.

Separation and Inactivation of Serum.—The blood is allowed to coagulate and the clot separated from the wall of the tube by loosening it with a stiff platinum wire, previously heated and cooled. In some laboratories, wooden applicators are employed for this purpose. This is not objectionable when the applicators are clean and are used only for one specimen. The tube is balanced on a scale against a tube containing a suitable amount of water and the two tubes are placed opposite each other in the centrifuge. Centrifugalization is carried on until there is complete separation of the serum from the coagulum. The serum is withdrawn with a capilary pipette fitted with a mouthpiece and rubber connection (Fig. 32, *k*) and placed in a clean tube, properly labelled. If centrifugalized one day and separated on the following morning, it is usually possible to pour the serum from the clot and by employing care, to keep it free from cells and hemolysis. This effects a considerable saving of pipettes. The serum is inactivated by immersing the tube in the water-bath to a depth equal to at least one-half its length, keeping at a temperature not below 55° C. and not above 58° C. for thirty minutes. Electrically heated baths are convenient. If a large quantity of serum is to be inactivated, it should be divided between two or three tubes. When the reactions are to be set up, 1 cc. is placed in a clean tube and 4 cc. of physiological saline solution are added to make a 1:5 dilution.

Reagents.—1. *Salt Solution.*—Physiological saline solution (0.85 or 0.9 per cent.).

2. *Sheep Cells.*—Suspension (5 per cent.) of sheep's red blood corpuscles.

3. *Hemolytic Amboceptor.*—Serum of rabbit, immunized against sheep corpuscles.

4. *Complement.*—Pooled serum of guinea-pig's blood.

5. *Antigen.*—Cholesterolized (0.4 per cent.) alcoholic extract of human, beef or guinea-pig heart.

Dilution of reagents is made conveniently in graduated glass stoppered cylinders (Fig. 32, *c*).

Physiological Saline Solution.—This should be prepared from chemically pure sodium chloride and freshly distilled water. The salt must be weighed carefully. Too small an amount will give an hypotonic solution which hemolyzes red cells promptly. A markedly hypertonic solution will inhibit hemolysis. Since in spite of care mistakes are sometimes made, it is well to make up each batch of salt solution in advance of the time when it will be used, and to test it out carefully. When 2 cc. are added to 0.5 cc. of 5 per cent. red cell emulsion, hemolysis should not occur.

It is advisable to sterilize the flasks of saline if they are to be kept on hand for more than a few days. The level of the fluid in the flask should be marked on the side with a file scratch, and sterilization accomplished either by boiling for five minutes or by treatment with 15 pounds of steam pressure in the autoclave for fifteen minutes. In either case the saline should be cooled and the lost volume restored to the mark by sterilized distilled water.

Sheep-cell Emulsion.—Sheep's blood may be obtained by keeping a sheep and by withdrawing blood from the jugular by means of a syringe when desired. The inconvenience incident to keeping an animal of this size in the laboratory and of bleeding it are objectionable to some workers. Added to this is the fact that in time an anemia is produced if any considerable quantity be withdrawn regularly, and consequently the cells become less resistant. If an abattoir be conveniently located, it is usually possible to procure the services of an employee at small cost who will secure fresh blood from a healthy sheep and bring it to the laboratory.

The blood may be transported in a Mason jar, partially filled with sterile physiological saline to which has been added 2 per cent. sodium citrate. In this 5 volumes or less of sheep blood may be received and gently shaken to ensure adequate mixing. Another method is to provide a stout glass bottle (8 or 16 oz. capacity) with a wide "salt-mouth" and a good stopper, into which have been placed about two dozen pieces of glass rod or other bits of glass. This outfit should be sterilized by dry heat. When the blood is obtained in this outfit, it should not fill the bottle completely and it should be shaken vigorously for ten to fifteen minutes to obtain complete defibrination. The butcher should be instructed not to take the first blood from the animal but to let this run away in order to secure blood as free from animal's wool and infective material as possible.

The desired quantity is placed in centrifuge tube and centrifugalized until the cells are packed at the bottom of the tubes, when the supernatant fluid consisting of serum and diluent is removed by pipette and discarded. The tubes are then filled with sterile physiological saline with which the corpuscular mass is thoroughly mixed and again centrifugalized. This supernatant saline is again removed by pipette or by pouring and the process repeated at least twice. If the reactions are to be performed the same day, the suspension may be prepared at once, but if they are to be done later the cells should be kept in the refrigerator undisturbed under the fluid of the last washing in corked tubes until needed.

It is claimed that cells may be kept a long period of time even with only these precautions. With this the writer does not agree, unless, possibly, especial care is taken in procuring them. It is to be remembered that the cells are living, and that as soon as they die they become worthless, even though they be uninfected. Our experience has been that in cold weather the reactions should be done within forty-eight hours of the time when the sheep was bled, and that in the hot summer months the reactions should be done on the following day. If kept longer, the cells are apt to turn purple and hemolyze very easily while standing for the final reading.

When ready to prepare the suspension, the supernatant salt solution is removed and packed cells are measured with a graduated 1 cc. pipette into the desired quantity of salt solution. It is apparent that this suspension is bound to be inaccurate since the density of the sediment will vary with the speed of the centrifuge and other factors. A constant standard suspension is obviously much to be desired. It is convenient to standardize with a Sahli hemoglobinometer. With the standard tube of the hemoglobinometer filled with a mixture adjusted to give a reading of 100 per cent. with normal male blood, place $\frac{N}{10}$ HCl in the graduated tube to the 20 mark, add to it 0.5 cc. of the sheep corpuscle emulsion, mix, allow to stand one minute and then add distilled water to make the diluted fluid match the standard, as in performing the hemoglobin test on human blood. The reading should be 100 per cent. Cells or salt solution may be added to the sheep suspension as required to bring to standard.

Occasionally sheep corpuscles are obtained which are either more or less resistant to the action of the other members of the hemolytic system than usual. It has been our experience to encounter the former phenomenon occasionally. If increased resistance be well marked, it is advisable to secure fresh blood; if not marked, the strength of other members of the system may be adjusted to compensate.

Preparation of Hemolytic Amboceptor.—(Serum of rabbit immunized against sheep corpuscles.) A healthy, well grown rabbit should be selected. The sheep cells should be obtained with as great regard to sterility as possible. They are "washed" with sterile physiological saline at least four times, as directed for the preparation of sheep-cell suspension, and finally freed of saline solution. The cells should be warmed to approximately body temperature by placing the containing tube in water at 40° C.

Intravenous Injections.—A small sterile glass syringe fitted to a well sharpened needle of fine gauge is charged with cell emulsion. An assistant may hold the animal across his lap stretching the body by drawing the forelegs forward with one hand and the hind legs back with the other, or the animal may be wrapped in a pillow-slip, leaving the head out (Fig. 38). The operator draws the ear down and injects the blood slowly into the marginal vein.

A rabbit box is very convenient and renders an assistant unnecessary. This can be made quite simply by putting a hole in the top of a box about 16 inches long by 6 wide and 7½ deep. The hole is for the rabbit's neck, the head being outside the box, the body inside. This top is made in two pieces to permit withdrawal of one part so that the rabbit may be placed in the box (Fig. 42).

The following schedule of injections may be followed:

Injection.	Washed undiluted sheep corpuscles.
First	1.0 cc.
Second	1.5 "
Third	2.0 "
Fourth	2.0 "

The time-interval should be three to five days between the injections. Although it may be lengthened to seven days there is some danger of producing anaphylactic shock. Exactness of dosage is not a rigid requirement.

Intraperitoneal Injections.—If preferred, the injections may be given into the peritoneal cavity. This has no especial advantage except possibly that the technic of injection is easier. Larger doses of cells must be given, however, and there is greater danger of infection and consequently a higher mortality. A stouter needle should be used than that prescribed for the intravenous method. The animal is held by the assistant with head down, as shown in the illustration (Fig. 41).

Fig. 38

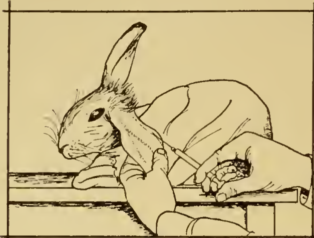
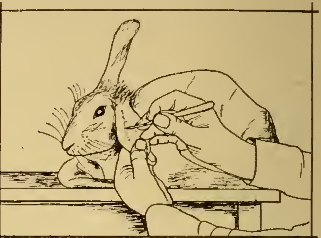


Fig. 39



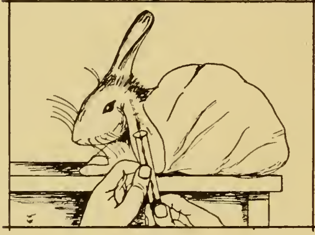


Fig. 40

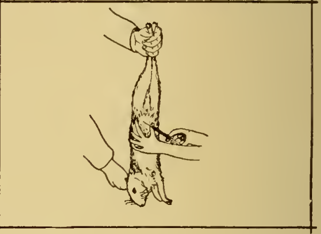


Fig. 41

Fig. 38.—Injecting cells into lateral ear vein of rabbit. Rabbit wrapped in pillow-slip.

Fig. 39.—Making incision into central ear vein.

Fig. 40.—Collecting specimen of rabbit's blood for preliminary amboceptor titer.

Fig. 41.—Injecting cells intraperitoneally.

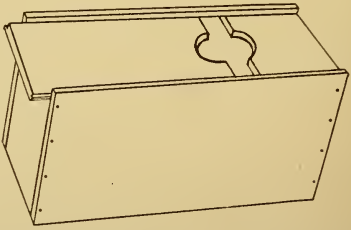


Fig. 42.—Rabbit box, showing sliding top and hole for animal's head.

The hair is washed with alcohol and the needle is inserted in the median line a little below a point midway between ensiform cartilage and the pubis. A small spot may be shaved, but this is really not necessary, since the hair may be separated and "plastered down" with the alcohol. The cells should be warmed and injected slowly. Injections may be given according to the following schedule.

Injection.	Washed sheep corpuscles diluted 50 per cent.
First	5 cc.
Second	10 "
Third	10 "
Fourth	10 "

Untoward Symptoms.—In giving injections intravenously, untoward symptoms may develop if cells are injected so rapidly that the right heart is overloaded. With any mode of injection, anaphylactic symptoms may appear, particularly with longer time intervals. It is worth while to attempt saving the rabbit by an intravenous injection of 1 to 2 mm. adrenalin chloride (1:1000) solution.

Preliminary Titer.—The titer of the serum may be determined at any time. It is the writer's practice to give 4 injections by the intravenous route, then to wait seven days, secure blood and do a preliminary titer. Almost invariably serum of high potency is obtained with 3 or 4 injections. If the titer is low, an additional injection may be given. Blood is obtained easily by making a small incision with a small, sharp-pointed scalpel over the *median* vein of the ear, carrying the incision through the skin and across the vein, allowing 2 or 3 cc. of blood to run into a test-tube (Figs. 39 and 40). The animal may be held across an assistant's lap as for intravenous injections. Bleeding is stopped by making firm pressure over the incision for a few moments with a pledget of cotton. The blood in the tube is allowed to clot and is centrifugalized, when the serum is removed and inactivated at 56° C. for thirty minutes. It is now ready for titration. For titration complement should be used from pooled guinea-pig's serum which has been titrated against an already standardized hemolytic amboceptor. A 5 per cent. emulsion of sheep corpuscles is used, standardized as specified above. A row of 12 tubes is set up in a test-tube rack, placing a single tube in the rear row as a control (Table A). In the first tube is placed 0.8 cc. of physiological saline and in each of the remaining eleven in the front row is placed 0.5 cc. To the first tube is added 0.2 cc. of the rabbit serum to be tested. For this purpose a 1 cc. pipette graduated in 0.01 cc. should be used. In the first tube we now have 0.2 cc. of immune serum and 0.8 cc. of salt solution, the total being 1.0 cc. The pipette is emptied, the mixture in the first tube is drawn into the pipette and blown back into the tube 2 or 3 times to ensure thorough mixing and finally 0.5 cc. is placed in tube No. 2. It is evident that we have left in tube No. 1 0.5 cc. of mixture and that there is present 0.1 cc. of immune serum (or one-half of the amount put in at first). We have carried into tube No. 2, 0.5 cc. of the mixture, which also contains 0.1 cc. of immune serum. The contents of tube No. 2 are thoroughly mixed as before and 0.5 cc. of the mixture is put in tube No. 3. Now we have only 0.05 cc. of undiluted serum left in tube

No. 2. The process of mixing is repeated with tube No. 3 and its contents divided, and so on until tube No. 12 is reached. When tube No. 12 has received one-half the contents of tube No. 11, mixture is made and 0.5 cc. of the resultant mixture discarded. The quantities of *undiluted* immune serum are shown in the table. To each tube is now added 0.25 cc. of complement dilution (prepared so that this amount will contain one unit), 0.5 cc. of sheep corpuscle emulsion, and 1.25 cc. of physiological salt solution. The control tube is set up by putting in 0.1 cc. of undiluted immune serum, 0.5 cc. of sheep corpuscle emulsion, and 1.9 cc. of normal salt solution. The rack is placed in the water-bath at 37° C. for one hour shaking the tubes every ten to fifteen minutes. The reading is then taken, that is, the last tube is noted in which complete hemolysis has taken place. The amount of immune serum in this tube represents *one unit* of hemolytic amboceptor. Of course there should be no hemolysis in the control tube.

Ordinarily hemolysis should be complete at least in tube No. 8, that is, the serum should be so strong that about 0.0006 cc. or less will contain one unit, and it is our custom to secure sera of which 0.00006 cc. to 0.0003 cc. or less will contain one unit. If the strength of the serum be sufficient, the animal may be bled to death. Of course this titration is crude, the results are only approximate and it is meant only to determine whether or not immunization has been successful. Such a titration is not accurate enough to permit its use in finally determining the dilution of the amboceptor previous to its actual use in the reaction. There should be no hemolysis in control tube No. 13 because it contains no complement. In the hypothetical instance shown in Table A, one unit is 0.00019 cc., since this is the least amount which shows complete hemolysis.

TABLE A.—PRELIMINARY TITER OF THE HEMOLYTIC AMBOCEPTOR.

Tube No.	Actual amount of undiluted immune rabbit serum, cc.	Additional physiological saline, cc.[1]	Amount of diluted complement (= 1 unit), cc.	Amount of 0.5 per cent. sheep corpuscle emulsion, cc.		Results in hypothetical instance.[2]
1 . . .	0.1	1.25	0.25	0.5	In water-bath at 37° C. for one hour.	C.H.
2 . . .	0.05	1.25	0.25	0.5		C.H.
3 . . .	0.025	1.25	0.25	0.5		C.H.
4 . . .	0.0125	1.25	0.25	0.5		C.H.
5 . . .	0.0062	1.25	0.25	0.5		C.H.
6 . . .	0.0031	1.25	0.25	0.5		C.H.
7 . . .	0.00155	1.25	0.25	0.5		C.H.
8 . . .	0.00078	1.25	0.25	0.5		C.H.
9 . . .	0.00039	1.25	0.25	0.5		C.H.
10 . . .	0.00019	1.25	0.25	0.5		C.H.
11 . . .	0.000095	1.25	0.25	0.5		P.H.
12 . . .	0.000042	1.25	0.25	0.5		N.H.
13 . . .	0.1	1.9	0	0.5		N.H.

[1] This amount is in addition to that originally placed in the tubes for the purpose of fractionating the immune serum.

[2] C.H. means complete hemolysis; P.H., partial hemolysis; and N.H., no hemolysis.

Bleeding the Rabbit.—First Method.—The anterior surface of the rabbit's neck and the upper part of the chest is lathered with shaving soap, shaved and washed with alcohol and ether. A gauze pad soaked with alcohol is held in place over this area by an assistant. The fore legs are tied together with a stout cord, one end of which should be passed to a second assistant who is to hold the animal. The hind legs are also tied together. In this way one assistant is able to hold back both pairs of legs. The animal is now turned belly down, the first assistant holding the head out on a stretch and etherizing the animal, the second assistant aiding by pulling the fore and hind legs posteriorly and steadying the body. When etherized, the animal's neck is held over a wide sterile funnel inserted into a sterile 50 cc. centrifuge tube. The alcohol-soaked pad is removed, the neck dried with sterile gauze and the vessels of the neck cut with a quick sweep of a sharp amputating or autopsy knife, previously sterilized by prolonged immersion in 95 per cent. alcohol and dried with sterile gauze. A second sterile 50 cc. centrifuge tube should be standing in readiness in the rack, since a little over 50 cc. of blood are usually secured. When the animal is bled out, the tubes of blood are corked with sterile corks and placed in the refrigerator.

Second Method—The rabbit is tied down on a board, etherized, and a long incision made through the skin, dissecting out the carotid artery under sterile conditions. The artery is tied off and nicked but not cut entirely across on the heart side of the ligature. The blood is allowed to spurt into a sterile wide mouth tube. Some workers insert a fine cannula into the artery. While this method is theoretically irreproachable on grounds of asepsis, longer etherization is required and the mortality is higher. It is somewhat more difficult technically and etherization reduces the titer of the amboceptor.

Third Method.—The animal is tied down on a rabbit board. The hair is plucked from the inguinal region, the skin sterilized by applying alcohol, and a long incision made through it over and parallel to Poupart's ligament. The edges of the incision are retracted and a cut made through the fascia, which is carefully separated. This is an important step in the procedure. Then the femoral vessels are cut with the knife and the blood is allowed to flow into a sterile 50 cc. centrifuge tube or Erlenmeyer flask, pressing the mouth against the exposed tissues close to the bleeding vessels. Etherization is unnecessary and the operation may be performed quickly. The only instruments required are sterile knife and dissecting forceps.

Preparation and Preservation of Hemolytic Amboceptor.—It is preferable to allow the blood to stand in the refrigerator over night since the serum gains slightly in potency. On the following day it may be separated exactly as in the manner described in the preparation of human blood with a sterile capillary pipette. The bulk of the serum is divided among 3 or 4 sterile test-tubes (size $\frac{3}{4}$ x 6 inches), while into each of 7 or 8 small tubes (size $\frac{3}{8}$ x 4 inches) are placed 0.5 cc.

The latter amounts must be measured out with a 1 cc. pipette, graduated in 0.1 cc. All of the tubes are sealed in a blowpipe or the hot flame of a Tyrill burner. The smaller tubes are for use in the preliminary titrations. As the need arises the larger tubes may be opened and divided up into small tubes containing 0.5 cc. each. It is wise to keep all tubes in the ice-box. In some laboratories it is the custom to keep the serum in bulk in a bottle from which serum is pipetted whenever needed. This incurs the not inconsiderable risk of contaminating a valuable reagent.

When amboceptor is desired, one of the small sealed tubes containing 0.5 cc. is opened at the top by nicking with a sharp file, removing the upper portion with a sharp blow. Into the tube is placed 4.5 cc. of physiological salt solution, which gives an ample quantity of 1:10 dilution for titration and the day's reactions. Immune hemolytic serum may be preserved in this manner for many months with no change in the titer.

TABLE B.—TABLE SHOWING TITER OF HEMOLYTIC AMBOCEPOTR.

Tube No.	Amboceptor in dilution to be used.	Amount of dilution. cc.	Amount of 0.9 per ct. NaCl solution, cc.	Amount of diluted complement (= 1 unit), cc.	Amount of 5 per cent. sheep corpuscle emulsion, cc.		Result in hypothetical case.	Actual amount of undiluted amboceptor, cc.
1 . .	1:10	0.1	1.65	0.25	0.5	In water-bath at 37° C. for one hour.	C.H.[1]	0.01
2 . .	1:100	0.1	1.65	0.25	0.5		C.H.	0.001
3 . .	1:1000	0.5	1.25	0.25	0.5		C.H.	0.0005
4 . .	1:1000	0.4	1.35	0.25	0.5		C.H.	0.0004
5 . .	1:1000	0.3	1.45	0.25	0.5		C.H.	0.0003
6 . .	1:1000	0.25	1.50	0.25	0.5		C.H.	0.00025
7 . .	1:1000	0.2	1.55	0.25	0.5		C.H.	0.0002
8 . .	1:1000	0.15	1.60	0.25	0.5		C.H.	0.00015
9 . .	1:1000	0.1	1.65	0.25	0.5		M.H.	0.0001
10 . .	1:10000	0.9	0.85	0.25	0.5		M.H.	0.00009
11 . .	1:10000	0.8	0.95	0.25	0.5		P.H.	0.00008
12 . .	1:10[2]	0.1	1.90	0	0.5		N.H.	0.01

Final Determinations of the Hemolytic Amboceptor Titer.—Before finally deciding upon the final titer of a new amboceptor, it should be titrated on several occasions, using complement from pooled guinea-pig sera the unit of which has been determined by titration against a tried amboceptor. Having prepared a 1:10 dilution of amboceptor as directed in the preceding section, a 1:100 dilution should be made from this by mixing 1 cc. of the 1:10 dilution with 9 cc. of physiological saline. From this in turn is made a 1:1000 dilution by taking 1 cc. (of the 1:100 dilution) and adding to 9 cc. of saline. Likewise from this is made a 1:10,000 dilution by taking 1 cc. (of the 1:1000 dilution) and adding the 9 cc. of saline. We will assume that the preliminary titer showed that one unit was equal to about 0.00019 cc. A rack of tubes would be set up as indicated in Table B, putting into each the amount of diluted hemolytic serum specified of the dilution specified and adding 0.25 cc. of diluted complement (containing one unit of complement), 0.5 cc. of sheep-cell emulsion, and physiological saline solution sufficient to bring the total volume in each tube to 2.5 cc.

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[1] C.H. means complete hemolysis; M.H., marked hemolysis; P.H., partial hemolysis; N.H., no hemolysis.

[2] Tube No. 12 is the control and should show no hemolysis.

According to the table, it is seen that one unit of amboceptor, that is, *the least quantity which will hemolyze the sheep corpuscles in the presence of one unit of complement,* is equal to 0.00015 cc. The titration is repeated on three or four occasions and the average of the results taken. When the amboceptor is finally used in performing the reaction, it must be diluted so that 0.5 cc. will contain 2 units. The following equation will be of assistance in determining the amount of saline solution to be added to a given quantity of 1:10 dilution of hemolytic amboceptor:

X represents the total quantity of diluted amboceptor.
A represents the amount of *undiluted* amboceptor which is used.
0.5 is the amount of the *diluted* amboceptor which we desire to contain 2 units.
B represents the quantity of undiluted amboceptor equivalent to 1 unit.

$$X : A = 0.5 : 2B.$$

Example.—Suppose that we desire to start with 1 cc. of our *1:10 dilution* of amboceptor (or 0.1 cc. of undiluted amboceptor) and that the titer shows 1 unit equals 0.00015 cc., as illustrated above:

$$X : 0.1 = 0.5 : 2(.00015)$$
$$X = 166.7$$

That is, 1 cc. of 1:10 dilution (equivalent to 0.1 cc. of undiluted amboceptor) is to be diluted up to a total volume of 166.7 cc. with physiological salt solution. In other words, we desire a dilution of 1 to 166.7. We may check the correctness of our calculation in this way. With 166.7 cc. of diluted amboceptor, we have 333.4 doses of diluted amboceptor 0.5 cc. each. Each dose, therefore, would contain $\frac{1}{333}$ of undiluted amboceptor (0.1 cc.), or .0003 cc., which is what is desired.

In titrating complement titer before each day's work, it is advisable to repeat the titer as a check on the indicator system for while the amboceptor itself is almost stable, such a titer serves as an excellent supplement to the complement titer. If desired, several of the tubes may be omitted, leaving those which are near the hemolytic unit.

Preparation and Titration of the Complement.—Complement is obtained from guinea-pig blood. The use of pooled serum from three or four pigs weighing 400 gm. or more is advisable since specimens from different pigs vary considerably. In order to prevent dilution of the blood by hydremia, the pigs may be fed on an exclusive diet of oats for at least a day before they are bled. It is poor economy to use small pigs, since the yield of blood is scanty and weak in complement.

Serum may be procured either by cutting the animals' throats and bleeding them to death, or by aspirating blood with a syringe from the right auricle. The latter method has the advantage of economy, since the pig may be kept alive for some time to furnish many small lots of complement, but an anemia is produced resulting in weak complement.

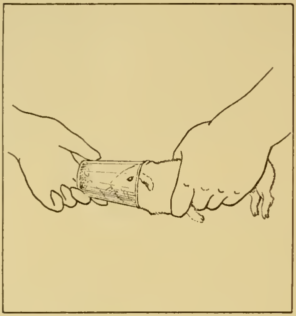

FIG. 43.—Etherizing guinea-pig.

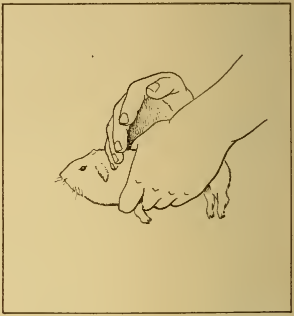

FIG. 44.—Stunning pig with sharp blow in occipital region.

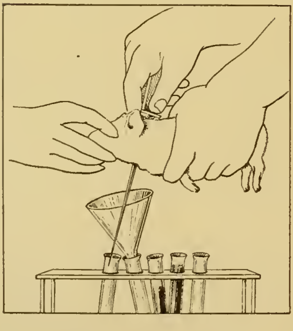

FIG. 45.—Cutting throat.

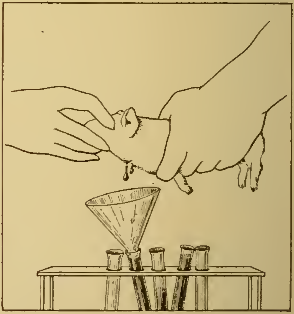

FIG. 46.—Bleeding through a funnel into test-tube.

Methods for Obtaining Blood.—(*a*) The animals may be bled into Petri dishes, but preferably through a funnel into test-tubes, since in the latter way centrifugalization and separation of the serum is facilitated. In a test-tube rack are placed several tubes which will fit the centrifuge and into one is placed a wide funnel. The animal may be etherized lightly (Fig. 43), or stunned by a sharp blow at the base of the skull with the ulnar side of the hand (Fig. 44). An assistant draws the animal's head forward, the operator holds the fore-paws out of the way and pulls the body back, and severs the vessels of the neck with a long knife (Fig. 45) while the neck is held over the funnel (Fig. 46). (*b*) A needle about 1 inch long of 18 gauge is attached to a glass syringe (Fig. 32, *j*) of Luer type. The pig is etherized lightly and the hair clipped off in the precordial area. It is held firmly, preferably by an assistant. The point of maximum cardiac pulsation is selected, the skin sterilized with iodine, and the needle inserted between the ribs at this point, withdrawing about 5 cc. of blood (Fig. 47).

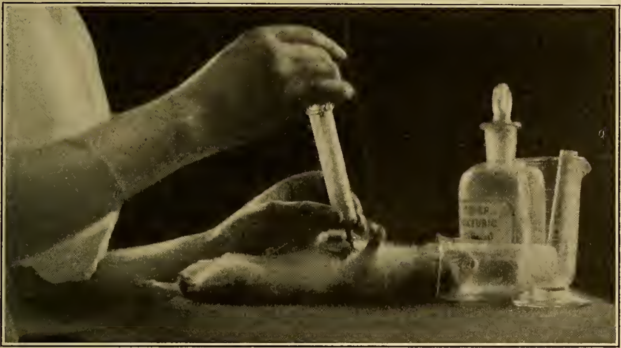

Fig. 47.—Method of bleeding guinea-pig for complement. (Thompson.)

Separation and Care of Serum.—When possible, the serum is allowed to stand on the clot for several hours, preferably over night. It may be separated in the manner directed for human blood serum (page 128). The serum for all tubes should be mixed, preferably in a glass-stoppered graduated cylinder.

Especial care must be taken to keep the complement cold at all times except when in actual use. Both before and after separation and again after final dilution, it should be placed in the refrigerator. It should not be shaken, but may be mixed by inverting the glass cylinder. The worker should remember that complement is unlike amboceptor in that it is thermolabile and readily deteriorates at higher temperature.

Titration of Complement.—In titrating the complement, amboceptor of known strength should be used and diluted so that 0.25 cc. of the dilution contains 1 unit. A 5 per cent. sheep corpuscle emulsion should be prepared and standardized as directed (page 129) and a 1:10 dilution of pooled complement prepared by adding 9 cc. of physiological saline to 1 cc. of serum. The titer is set up as indicated in

Table C, placing graduated amounts of complement dilution in the tubes, each receiving 0.25 cc. hemolytic amboceptor dilution, 0.5 cc. sheep corpuscle emulsion, and sufficient physiological saline to bring the total volume to 2.5 cc. Three controls are necessary, one containing amboceptor, corpuscles and saline only, to determine that the amboceptor does not produce hemolysis without complement; one with complement, corpuscles and saline only, to find out whether or not the complement itself contains a hemolytic substance; and another with corpuscles and saline only, to make certain that the cells do not hemolyze spontaneously.

TABLE C.—TABLE SHOWING COMPLEMENT TITER.

Tube No.	Amount of 1:10 complement dilution, cc.	Amount of amboceptor in dilution (0.25 cc. = 1 unit), cc.	Amount of 5 per cent. sheep corpuscle emulsion, cc.	Amount of 0.9 per cent. NaCl solution, cc.		Results in hypothetical case.	Actual amount of undiluted complement, cc.
1 . . .	0.5	0.25	0.5	1.25	In water-bath at 37° C. for one hour.	C.H.[1]	0.05
2 . . .	0.4	0.25	0.5	1.35		C.H.	0.04
3 . . .	0.3	0.25	0.5	1.45		C.H.	0.03
4 . . .	0.25	0.25	0.5	1.50		C.H.	0.025
5 . . .	0.2	0.25	0.5	1.55		P.H.	0.02
6 . . .	0.1	0.25	0.5	1.65		S.H.	0.01
Control 1	0	0.25	0.5	1.75		N.H.	0.0
Control 2	0.5	0	0.5	1.50		N.H.	0.05
Control 3	0	0	0.5	2.00		N.H.	0.0

According to the illustrative table, it will be seen that one unit of complement, that is, the least quantity which is sufficient to cause complete hemolysis of a unit of sheep cells in the presence of an excess of hemolytic amboceptor is 0.25 cc. of 1:10 dilution, or 0.025 cc. of undiluted complement.

If the titer be set up with 2 units of amboceptor in each tube, the results when read at the end of ten minutes' incubation at 37° C. will be very similar to those obtained with the use of 1 unit in each tube, read at the end of sixty minutes.

It is the author's practice to take advantage of this fact, setting up a "short" titer with 2 units of amboceptor in each tube, and obtaining a preliminary result in ten minutes. With this reading as an indication of the final result, a second titer is prepared using one unit of amboceptor in each tube. The accuracy of the determination is enhanced by employing a 1:20 dilution of complement instead of the customary 1:10 dilution. If, for example, the "short" titer showed one unit of complement to be approximately 0.2 cc., the final titer would be set up with 0.46 cc., 0.44 cc., 0.42 cc., 0.40 cc., 0.38 cc., 0.36 cc., 0.34 cc., 0.32 cc. of 1:20 complement dilution, corresponding respectively to 0.23 cc., 0.22 cc., 0.21 cc., 0.19 cc., 0.18 cc., 0.17 cc., 0.16 cc. of 1:10 dilution.

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[1] C.H., means complete hemolysis; P.H., partial hemolysis; S.H., slight hemolysis; N.H., no hemolysis.

Interpretation of the Results.—A complement should not be employed which is so weak that it will not produce complete hemolysis in tube No. 2 (that is, with 0.04 cc. undiluted complement), or which is so strong that it produces complete or nearly complete hemolysis in tube No. 6 (that is, with only 0.01 cc. undiluted complement). A complement is not to be employed which shows hemolysis in control tube No. 2.

This titer having been completed, a small amount of complement is diluted accordingly, and a set of trial reactions is prepared. These serve as a check on the balance between the members of the hemolytic system, as will be explained later (for number of units to be employed, see page 147).

In diluting the complement, the following equation may be helpful:

X represents the total quantity of diluted complement.
A represents the amount of undiluted pooled serum to be used.
0.5 cc. is the amount of complement dilution which we desire to contain 1.5 units.
B represents the quantity of complement found on titration to be 1 unit.

Example.—

$$X : A = 0.5 : 1.5B.$$

Now suppose that we wish to dilute 8 cc. of guinea-pig serum, and that the titer shows (as in above table) that 1 unit =0.025 cc.

$$X : 8 = 0.5 : 1.5\ (0.025)$$
$$X = 106.6$$

Then 8 cc. of serum are diluted by adding 98.6 cc. of physiological salt solution to make the total volume 106.6 cc.

The Antigens.—Preparation.—Cholesterolized Alcoholic Extracts of Normal Organs.—Human, beef, or guinea-pig hearts may be used. Human heart should be obtained preferably from an accident case or at least one free from infection, and as soon after death as possible. Guinea-pig hearts may be removed from animals which have been bled out to secure complement, prepared as directed in the following paragraph and placed with a suitable amount of alcohol in a bottle. Additional hearts with proportional amounts of alcohol may be added from time to time.

The heart-tissue should be freed from blood, using only myocardium and removing fibrous and fatty tissue. The desired amount is weighed, cut into small pieces, and ground in a clean dry mortar with bits of clean broken glass. The resulting paste-like mixture is taken up with a spoon and put into a clean Erlenmeyer flask or Mason jar. Absolute alcohol should be added in the proportion of 100 cc. of alcohol to each 10 gm. of heart. The receptacle should be tightly stoppered and kept in the incubator at 37° C. about seven days when it is cooled by placing in the refrigerator over night. After cooling, the extract is filtered through fat-free filter paper and the filtrate kept in a dark bottle in the refrigerator. To 100 cc. is added 0.4 gm. cholesterol (C. P.). This is dissolved in the antigen by placing it in the incubator at 37° C. over night when the solution is cooled at room temperature and filtered, if any sediment be seen. Cholesterolized antigens are of remarkably uniform strength.

Alcoholic Extract of Syphilitic Liver.—A liver from a syphilitic fetus is obtained in as fresh a condition as possible and washed free from blood. A portion is weighed and ground in a mortar with a pestle with pieces of broken glass. The resulting mass is placed in a Mason jar or a wide mouthed flask or bottle and to it is added absolute alcohol in the proportion of 100 cc. of alcohol to each 10 gm. of liver tissue. The mixture is placed in the incubator from seven to ten days, shaking well each day, when it is filtered through filter paper. The filtrate should be titrated. If the antigenic strength proves insufficient, the filtrate may be evaporated to two-thirds its original volume by placing it in an open dish in front of an electric fan. (Craig.)

Noguchi Acetone—Insoluble Antigen.—Normal fat-free heart-muscle is ground up and absolute alcohol added (100 cc. alcohol to 10 gm. tissue). This is kept at 37° C. for seven days, shaking each day, and then filtered. The filtrate is evaporated to dryness with an electric fan. This step ordinarily requires fifteen to twenty-four hours. The residuum is dissolved in ether and the opalescent ethereal extract allowed to stand in the refrigerator over night so that the sediment may settle. In the morning, the clear supernatant fluid is poured off and evaporated in front of an electric fan to small bulk. *Care should be taken to avoid igniting the ether.* Approximately 10 volumes of chemically pure acetone are added to the residuum. The acetone mixture is put aside for several hours and the acetone decanted. In this way cholesterol is discarded, since it is acetone-soluble; and the sticky brown precipitate, made up of lecithin and certain acetone-insoluble lipoids, is collected with a spatula and kept in a bottle under acetone.

To prepare for use, about 0.3 gm. of the acetone-insoluble precipitate is dissolved in 1 cc. of ether and 9 cc. of methyl alcohol are added. This may be kept as stock solution and a small quantity diluted 1:10 with physiological saline as required for the test.

Rapid Method of Preparing Antigens from Normal Heart Muscle.—Ecker and Sasano adopt the suggestions of Erlandsen and of Neymann and Gager to extract finely divided heart muscle, dried in air, with ether and then alcohol, discarding the ethereal extract which contains no substances of antigenic value except small quantities of lecithin which has high anticomplementary power. Normal heart tissue is freed from fat, larger bloodvessels, endocardium and pericardium, ground finely, spread on glass plates, and dried at incubator temperature or in front of an electric fan. The dried material is powdered in a mortar. This may be kept in bottles for some time. Twenty-five gm. are extracted 3 times, using 50 cc. of ether each time for a period of ten minutes. The ether is discarded and the heart tissue allowed to dry, when it is mixed with 75 cc. of 95 per cent. alcohol in a flask and extracted in a reflux condenser over a boiling water-bath for three hours. A reflux condenser may be made by inserting a 40-inch glass tube of 3 mm. diameter through the stopper of the flask, supporting this in a vertical position so that the condensed alcohol will run back into the flask. An antigen prepared in this way may be titrated in dilutions of 1:10, 1:50, 1:100, 1:150, 1:200, using 0.5 cc. of each dilution with known positive serum, and twice the quantity of each dilution with negative serum. Ecker and Sasano found that it was usually possible to employ these antigens in dilutions of 1:20.

Titration of the Antigen.—The following properties should be determined by titration: (1) hemolytic action; (2) anticomplementary action; (3) specific binding power. The balance of the hemolytic system must be properly adjusted and positive and negative sera must be on hand. The positive serum must be titrated as shown in Table D. A standardized antigen is desirable. The method of setting up the necessary titers is shown in Parts I to V of Table E.

TABLE D.—DETERMINATION OF SYPHILITIC UNIT OF POSITIVE SERUM PRELIMINARY TO TITRATION OF ANTIGEN.

Tube No.	Actual amount of syphilitic serum, cc.	Amount of standard antigen, cc.[1]	Additional 0.9 per cent. NaCl, cc.[2]	Amount of diluted complement (= 1.5 units), cc.		Amount of diluted amboceptor (= 2 units), cc.	Amount of sheep cell suspension (5 per ct.), cc.		Results in hypothetical case.
1 . .	0.1	0.5	0	0.5	In water-bath at 37°C. for one hour.	0.5	0.5	In water-bath at 37°C. for one hour.	++++
2 . .	0.05	0.5	0	0.5		0.5	0.5		++++
3 . .	0.025	0.5	0	0.5		0.5	0.5		++++
4 . .	0.012	0.5	0	0.5		0.5	0.5		++
5 . .	0.006	0.5	0	0.5		0.5	0.5		±
6 . .	0.003	0.5	0	0.5		0.5	0.5		—
7 . .	0.2	0	0.8	0.5		0.5	0.5		—

TABLE E, PART I.—DETERMINATION OF HEMOLYTIC QUALITIES.

Tube No.	Amount of antigen dilution (1:10), cc.	Amount of known negative serum, cc.	Amount of sheep-cell suspension, 5 per cent. cc.	Amount of 0.9 per cent. NaCl solution, cc.		Results in hypothetical case.
1 .	0.5	0.1	0.5	1.4	In water-bath at 37° C. for one hour.	N.H.[3]
2 .	1.0	0.1	0.5	1.9		N.H.

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[1] A known antigen, used and clinically tested, diluted according to previous titration, usually 1:10.

[2] This is amount in addition to that originally placed in the tubes for the purpose of fractionating the immune serum.

[3] N. H. means no hemolysis.

TABLE E, PART II.—DETERMINATION OF ANTICOMPLEMENTARY UNIT.

Tube No.	Amount of antigen dilution (1:10), cc.	Amount of 0.9 per ct. NaCl solution, cc.	Amount of complement dilution (=1.5 units), cc.		Amount of amboceptor dilution (=2 units), cc.	Amount of sheep cell suspension, 5 per cent., cc.		Results in hypothetical case.
3 . . .	0.5	0.5	0.5	In water-bath at 37° C. for one hour.	0.5	0.5	In water-bath at 37° C. for one hour.	C.H.[1]
4 . . .	1.0	0	0.5		0.5	0.5		C.H.
5 . . .	1.25	0	0.5		0.5	0.5		C.H.
6 . . .	1.5	0	0.5		0.5	0.5		C.H.
7 . . .	2.0	0	0.5		0.5	0.5		P.H. or N.H.

TABLE E, PART III.—DETERMINATION OF ANTIGENIC UNIT.

Tube No.	Amount of antigen dilution, cc.	Amount of diluted syphilitic serum (= 1 unit), cc.	Amount of 0.9 per cent. NaCl solution, cc.	Amount of complement dilution (=1.5 u.), cc.		Amount of amboceptor dilution (= 2 un.), cc.	Amount of sheep cell suspension (5 per ct.), cc.		Results in hypothetical case.
8 . .	0.05	0.1	0.9	0.5	In water-bath at 37° C. for one hour.	0.5	0.5	In water-bath at 37° C. for one hour.	C.H.[2]
9 .	0.10	0.1	0.8	0.5		0.5	0.5		P.H.
10 . .	0.20	0.1	0.7	0.5		0.5	0.5		N.H.
11 . .	0.30	0.1	0.6	0.5		0.5	0.5		N.H.
12 . .	0.40	0.1	0.5	0.5		0.5	0.5		N.H.
13 . .	0.50	0.1	0.4	0.5		0.5	0.5		N.H.

TABLE E, PART IV.—DEMONSTRATION THAT ANTIGEN IS SPECIFIC.

Tube No.	Amount of antigen dilution, cc.	Amount of normal serum, cc.	Amount of 0.9 per cent. NaCl solution, cc.	Amount of complement dilution (=1.5 u.), cc.		Amount of amboceptor dilution (= 2 un.), cc.	Amount of sheep cell suspension (5 per ct.), cc.		Results in hypothetical case.
14 . .	0.2	0.1	0.7	0.5	In water-bath at 37° C. for one hour.	0.5	0.5	In water-bath at 37° C. for one hour.	C.H.[3]
15 . .	0.4	0.1	0.5	0.5		0.5	0.5		C.H.
16 . .	0.5	0.1	0.4	0.5		0.5	0.5		C.H.

[1] N.H. means no hemolysis.

[2] N.H. means no hemolysis; P.H., partial hemolysis; and C.H., complete hemolysis.

[3] The standard antigen should be diluted so that 0.5 c.c. will contain the required dose.

TABLE E, PART V.—SERUM CONTROLS.

Tube No.	Amount of old standardized dilution, cc.[1]	Amount of diluted syphilitic serum (=1 unit), cc.[2]	Amount of normal serum, cc.	Amount of 0.9 per cent. NaCl solution, cc.	Amount of complement dilution (= 1.5 units), cc.		Amount of amboceptor dilution (= 2 units), cc.	Amount of sheep cell suspension (5 p. c.), cc.		Results in hypothetical case.[3]
17 . .	0.5	0.1	0	0.4	0.5	In water-bath at 37 C. for one hour.	0.5	0.5	In water-bath at 37 C. for one hour.	N.H.
18 . .	0	0.2	0	0.8	0.5		0.5	0.5		C.H.
19 . .	0.5	0	0.1	0.4	0.5		0.5	0.5		C.H.
20 . .	0	0	0.2	0.8	0.5		0.5	0.5		C.H.

Determination of the Unit of Syphilitic Serum —For this a series of 7 tubes are set up (see Table D). A serum should be selected which has given previously a strongly positive result. If it has been preserved for more than a day or so, it should be inactivated again for fifteen minutes. It is desirable to select a serum which does not contain native antisheep amboceptor. In the first tube (Table D) is placed 0.2 cc. of syphilitic serum and 0.8 cc. of physiological saline. In each of the next five are placed 0.5 cc. of physiological saline. The contents of the first tube are mixed thoroughly, and 0.5 cc. of the mixture is transferred to the second tube. The resulting mixture is drawn up into the pipette and ejected back into the tube several times to ensure thorough mixing, and 0.5 cc. is removed and put into the third tube. The process is repeated until the contents of six tubes are mixed, when 0.5 cc. are discarded. We now have six tubes containing respectively 0.1 cc., 0.05 cc., 0.025 cc., 0.0125 cc., 0.006 cc., and 0.003 cc., of syphilitic serum. The seventh tube is placed in the rear row of the rack and receives 0.2 cc., of undiluted syphilitic serum, 0.8 cc., of physiological saline and no antigen. Into each of the six tubes in the front row is placed the usual dose of the standard antigen. Into all seven tubes are placed 1.5 units of complement. The tubes are subjected to 37° C. for one hour and then 2 units of amboceptor and the usual amount (1 unit) of sheep cell suspension are added. They are again heated at 37° C. for one hour, when the results may be read. The least amount of syphilitic serum which causes complete inhibition of hemolysis (gives a ++++ reaction) is said to contain one syphilitic unit. In the hypothetical results given in the table, this occurs in tube No. 3, meaning that 0.025 cc. is one syphilitic unit. The serum should be diluted so that this amount will be contained in 0.1 cc., that is, to one part of serum is added three parts of physiological saline and 0.1 cc. of the mixture is used in titer.

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[1] This is the same serum as that used in Part III, diluted in the same manner.

[2] This is the same serum as that used in Part IV.

[3] N.H. means no hemolysis; C.H. means complete hemolysis.

Dilution of Antigen for Titration.—A 1:10 dilution should be made of cholesterolized antigen, and a 1:5 dilution of alcoholic organ extracts.

Discussion of Titration.—If the antigen has no hemolytic action in the quantities used in tubes 1 and 2 of Part I of the titration, it may be regarded as acceptable on this score. The anticomplementary unit shown in the hypothetical instance, Part II of the titration, Tube No. 7, is 2.0 cc. The dose of the diluted antigen to be used in the reaction may be that one-third of the amount (1.5 cc.) employed in tube No. 6, or one-third of the greatest amount which had no anticomplementary effect. This amount would be 0.5 cc. of a 1:10 dilution. That it is satisfactory from the standpoint of antigenic power is shown in Tube No. 10 of Part III of the titration, since so small an amount as 0.2 cc. of the 1:10 dilution causes complete fixation of the complement in the presence of *one* syphilitic unit. (The amount required here for complement fixation is considerably greater than would be needed if the syphilitic unit of the positive serum had not been determined and if the positive serum from an active case containing a number of syphilitic units had been employed. Had 0.1 cc. of serum of this sort been utilized in the antigen titer without titration of the syphilitic unit and without corresponding dilution, it would have a high binding-power and a new antigen would have shown complete fixation of the complement in much smaller amounts in the titer. It is evident that unless the syphilitic unit be ascertained, there will be *two* unknown quantities in the antigen titer, the syphilitic serum and the antigen to be titered). The dose, 0.5 cc. of a 1:10 dilution, which was selected because it was less than one-third of the anticomplementary unit, contains more than two antigenic units (the antigenic unit being 0.2 cc.). Part IV shows that the antigen is specific, since 0.5 cc. of a 1:10 dilution (the amount decided upon for use in the reactions), does not bind complement in the presence of 0.1 cc. of normal serum (tube No. 16). Of course the antigen should give negative results with a large number of normal specimens before it is actually employed in doing diagnostic reactions. The serum controls (Part V) are set up with a standard antigen to make certain that the normal serum employed is negative, (tube No. 19), and that the diluted syphilitic serum (tube No. 17) actually completely fixes the complement in the dilution employed. The latter is a cross check on the accuracy of the unit titer of the syphilitic serum. Tubes Nos. 18 and 20 are set up to correspond with the rear-tube control of the Wassermann reaction to show that neither positive nor negative serum has anticomplementary properties sufficient to interfere with the reaction. The result should be as indicated in the table.

To summarize, the amount of antigen employed in the test should be one-third of the amount which is anticomplementary or less; it should contain at least two antigenic units; and it should be less than one-half of the amount which hemolyzes sheep cells.

Dilution of Antigen.—When the antigen has been titrated and its properties ascertained, the worker is ready to determine the dilution. This to be made so that the required amount is 0.5 cc. of the emulsion. It is desirable to make the emulsion as strong as possible keeping it within the limits imposed by its anticomplementary titer (see above). In making the dilution, the required amount of physiological saline solution is measured out in a cylindrical graduate and the alcoholic antigen is added to it slowly, a drop or two at a time, mixing well after each addition of antigen. Other workers, Ruediger for example, advocate adding the salt solution slowly in small quantities to the antigen. The essential point is to follow the same method in diluting antigen for titration and for the reactions proper.

General Cautions.—In doing the reactions the worker must guard against non-specific complement-fixation. The hemolytic system should be carefully balanced. The human serum must be carefully inactivated, and a reading of the reaction should be not made unless the rear control tube for the reaction in question be clear. Positive controls should be positive and negative controls should be negative. An antigen may act queerly after a period of some months, either losing in strength or giving partial "false positives" on account of increased anticomplementary qualities. Mistakes on account of this may be avoided if all reactions are set up with three or four antigens, made at different times. The various properties of a constantly used antigen should be tested out by occasional titers.

It is not necessary to titer the antigens each time reactions are done, although the prescribed anticomplementary controls must be set up.

Controls and Preliminary Set of Reactions.—The directions given contemplate the use of an "indicator" (hemolytic) system made up of 1.5 units of complement and 2 units of amboceptor. Some workers recommend using 2 units of complement and 2 units of amboceptor when the cold fixation is used. This is probably the conservative course especially with cholesterolized antigens. The use of one unit of each reagent is unwise, since an excess of hemolytic substance is needed to overcome the anticomplementary action of human serum and antigen.

In performing Wassermann reactions, the use of reliable controls is absolutely essential. When work is being done regularly, this is not particularly difficult, since specimens may be kept from one reaction day to another. It is advisable to be certain that the negative control is really from a normal person. Specimens carried over from the preceding week should be again subjected to 56° C. for fifteen minutes to destroy any anticomplementary substances which may have developed on standing. An obviously infected serum should not be employed, since it may give a falsely positive result. Cloudiness of serum is sometimes due to high lipoid content. When the tube is held for a few moments in warm water, the opacity due to lipoids clears up, that due to bacteria remains. An especially desirable control is a positive spinal fluid. Spinal fluid keeps indefinitely if sterile, its antibody content does not change, and anticomplementary substances are not developed. A syphilitic unit titer should be made in the manner to be described later under examination of the spinal fluid (page 151) and this should be repeated as a control before setting up the day's reactions. With a properly balanced hemolytic system, the unit should be the same. When the preliminary reactions are set up, they should then include at least one positive and one negative blood serum from the preceding day's work, a positive spinal fluid of known titer, the antigen controls, and if possible a serum which gave a faintly positive result.

The antigen controls consist of two tubes for each antigen, one with double the amount and one with three times the amount of antigen used in the reactions. To each is added 2 units of complement, and after incubation, 2 units of hemolytic amboceptor and the regular amount of sheep corpuscles. The tubes are set up as directed below for the reactions proper. If the proper balance has been secured between the members of the hemolytic "indicator" system, the results should be the same as on the preceding day's work, *i. e.*, the negative should be negative, the strongly positive positive, the faintly positive as before, the spinal fluid giving a positive reaction with the same quantity as previously, and all the antigens controls showing complete hemolysis. If the formerly strongly positive reaction is not so strong, if the spinal fluid shows a negative reaction with the amount which should be positive, and a positive only with a high amount, and the blood (formerly faintly positive) is negative, then it is apparent that the complement is too strong, and its strength must be reduced. If, on the other hand, the negative blood gives a ± or a one plus result in any or all tubes, if the blood formerly faintly positive is strongly positive, if the spinal fluid shows complement-fixation with smaller quantity of fluid than usual, and if the antigens controls showed some impairment in hemolysis, the complement has been made too weak. *The spinal fluid is the best index.* Blood serum is more complex and occasionally the development of anticomplementary substances will render negatives falsely positive or, on the other hand, the deterioration of antibody due to preservation or re-inactivation allows positives to react negatively. It will be seen that the worker must interpret all his evidence judiciously and act accordingly. It is only by carrying out this preliminary work that the worker can do work, the results of which check from week to week.

Performance of the Reactions Proper.—A series of racks with double row of holes are prepared. If three antigens are to be employed, three tubes will be needed in the first row, and one control in the back row for each reaction. In each of the tubes in the front row is placed 0.5 cc. (containing 0.1 cc. undiluted serum) of the diluted inactivated serum to be tested, and 1.0 cc. (containing 0.2 cc. of undiluted serum) in the rear control tube (Table F). This is repeated for each serum to be examined. Positive and negative control sera are set up in the same way, and the antigen controls are prepared at the same time (page 146). Then to the tubes in front and back rows alike, is added 0.5 cc. (1.5 units) of complement dilution. In the *front* row of the reaction tubes is placed 0.5 cc. of antigen dilution, the first antigen being placed in the first tube of each reaction, the second in the second tube, etc. If warm fixation be employed, the racks are now placed in the water-bath at 37° C. for one hour, shaking occasionally (Fig. 36). If cold fixation be employed, they are kept at a temperature of 8 to 12° C. for four to twelve hours. When the required time has elapsed, into each tube, front and back alike, is added 0.5 cc. of hemolytic amboceptor dilution and 0.5 cc. of sheep corpuscle suspension and the racks are placed in the water-bath at 37° C. for one hour or until the antigen controls, the rear tube controls, and the negative serum controls have hemolyzed completely. It is important that during this time they be shaken frequently to prevent settling of the corpuscles to the bottom of the tubes.

TABLE F.—WASSERMANN REACTION ON BLOOD SERA.

Tube No.	Serum used.	Purpose of tube.	Amount of serum dilution (1:5), cc.	Amount of antigen dilution cc.	Amount of complement dilution (0.5 cc. = 1.5 un.), cc.		Amount of amboceptor dilution (0.5 cc. = 2 units), cc.	Amount of sheep corpuscle emulsion (5 per ct.), cc.		Result should be.
1 . .	A	For test	0.5	0.5	0.5	In water-bath at 37° C. for one hour.	0.5	0.5	In water-bath at 37° C. for one hour.	?
2 . .	A	Control on A test	1.0	0	0.5		0.5	0.5		C.H.[1]
3 . .	B	For test	0.5	0.5	0.5		0.5	0.5		?
4 . .	B	Control on B test	1.0	0	0.5		0.5	0.5		C.H.
5 . .	C	Positive control	0.5	0.5	0.5		0.5	0.5		++++
6 . .	C	Control on C serum	1.0	0	0.5		0.5	0.5		C.H.
7 . .	D	Negative control	0.5	0.5	0.5		0.5	0.5		C.H.
8 . .	D	Control on D serum	1.0	0	0.5		0.5	0.5		C.H.
9 . .	None	Control on antigen	0	1.0	0.5		0.5	0.5		C.H.
10 . .	None	Control on antigen	0	1.5	0.5		0.5	0.5		C.H.

This table provides for the use of only one antigen. When more are employed, additional front tubes (corresponding to 1, 3, 5 and 7) should be set up for each serum. Only one control tube (corresponding to 2, 4, 6 and 8) will be required for each serum. Two antigen control tubes (corresponding to 9 and 10) should be set up for each antigen employed in the test.

Detection of Native Antisheep Amboceptor.—If desired, each specimen of serum may be tested to determine the presence of native antisheep amboceptor during the first hour that the reactions are in the incubator or refrigerator.

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[1] C.H. means complete hemolysis; ++++, no hemolysis.

In each of a series of tubes is placed 0.25 cc. of a 1:5 dilution of human blood sera upon which Wassermann reactions are to be done and 0.25 cc. of complement dilution (one-half amount used for reactions), 0.5 cc. of cell emulsion, and 1.5 cc. normal saline. Place tubes in the incubator or water-bath at 37° C. for thirty minutes, shaking two or three times. When complete hemolysis results, it is evident that the serum contains enough native antisheep hemolytic amboceptor to complete the reaction so no further hemolytic amboceptor is added to the tubes in the reaction proper. By taking this simple precaution, one avoids the possibility of adding a great excess of unnecessary amboceptor. The native antisheep amboceptor may be removed from the serum if desired.

Removal of Native Antisheep Amboceptor.—To each 1 cc. of *inactivated* human serum is added 0.1 cc. of undiluted washed sheep cells. The mixture is shaken well, placed in the water-bath at 37° C. for thirty minutes, and then centrifugalized and the serum removed from the sedimented cells. The serum may be regarded as undiluted.

Reading the Reactions.—A preliminary reading may be taken as soon as the reactions are completed, at the end of the second incubation period. A final report, however, on these readings would be unsafe unless one has had much experience in such work.

A frank negative may be read at once. If an immediate report on any specimen is imperative, a doubtful reaction should be centrifugalized in order that the cells may be brought to the bottom of the tubes and the supernatant fluid be left cell-free for the detection of shades of hemolysis. The final readings are made usually after the reactions have stood two or three hours to permit partial settling of the cells. It is often convenient to allow them to stand in the refrigerator over night. Several criteria must be fulfilled before the reactions can be regarded as satisfactory. The positive control should give a positive result and the negative should be negative. All of the antigen controls must show complete hemolysis, and there should be almost universal complete hemolysis in the rear-tube controls. An occasional rear tube may fail to show hemolysis. This would be due probably to a poor specimen of serum, but if any marked number showing this condition, it is an indictment against the technic. When hemolysis is not complete in a rear-row control tube, that particular reaction must be disregarded, and another specimen of blood should be obtained from the patient.

In reading the results, the clarity or tinging of the supernatant fluid should be observed and the amount of residual sedimented cells noted.

The scale of readings is purely arbitrary. The writer employs one based on +++ representing a strongly positive result. The scheme proposed by Citron, however, is widely employed and will be given here. ++++ represents complete fixation of complement. There is no hemolysis. The result is *strongly positive.*

+++ represents 75 per cent. or more of complement-fixation, or 25 per cent. or less of hemolysis. The result is said to be *positive* or *moderately positive.*

++ represents 50 to 75 per cent. of complement-fixation, or 25 to 50 per cent. of hemolysis. The result is said to be *faintly positive.*

+ represents 25 per cent. or less of complement-fixation or more than 75 per cent. of hemolysis. The result is said to be *very faintly positive.*

± represents a faint rim of red cells only at the bottom of the tube. This is a *doubtful.* (It is usually negative.)

— represents complete hemolysis. The result is *negative.*

Wassermann Reaction on Spinal Fluid.—Since spinal fluid is free from complement and anticomplementary substances, inactivation is unnecessary. If free from blood and taken under sterile conditions in a sterile tube, it may be preserved in a refrigerator for a considerable period of time with no alteration in its antibody content and without the development of anticomplementary substances. Bacterial contamination, of course, will interfere with proper results.

In early work with the Wassermann reaction, the mistake was made of employing small quantities of spinal fluid, that is, it was used in the same amount as was blood serum. Larger quantities may be employed safely; and with them a much greater number of positive results is obtained in cases with definite syphilitic involvement of the central nervous system.

Measurement of the syphilitic antibody content of spinal fluid is extremely easy and should be done at least in all cases where treatment for cerebrospinal syphilis is contemplated, because observation of the change in the titer under treatment affords an index to progress. A series of tubes is set up, six in the front row of the test-tube rack and one in the rear. Into the front row tubes are placed respectively, 0.1 cc., 0.2 cc., 0.4 cc., 0.6 cc., 0.8 cc., and 1.0 cc., of spinal fluid (Table G). In the rear control tube is placed 2.0 cc. If there is insufficient fluid for all these tubes, the higher doses should be employed, always reserving enough for the rear control tube which should double the amount of the largest dose employed in the front row. To each front tube is added 0.5 cc. of antigen dilution and then to all tubes is added 0.5 cc. of complement dilution (containing 1.5 units). Physiological saline solution is added when necessary to bring the *final* volume to 2.5 cc. Fixation is carried out as with blood serum, either in water-bath or incubator at 37° C., for one hour, or at 8 to 12° C. from four to ten hours. Then 0.5 cc. of amboceptor dilution (containing 2 units) and also 0.5 cc. of 5 per cent. sheep corpuscle emulsion are added to each tube, front and back. The tubes are kept in the water-bath at 37° C. for one hour with frequent shaking, or until the negative controls, the antigen controls, and the rear-tube controls have entirely hemolyzed. In reading and reporting, the tube should be noted in which a positive result first appears. In the illustrative table giving hypothetical results, the report would be ++ with 0.4 cc., and ++++ with 0.6 cc. The rear control must show complete hemolysis or the result will have to be considered unsatisfactory.

TABLE G.—WASSERMANN REACTION WITH SPINAL FLUID.

Tube No.	Amount of spinal fluid, cc.	Amount of antigen dilution, cc.	Amount of complement dilution (0.5 cc. = 1.5 un.), cc.	Amount of 0.9 per cent. NaCl solution, cc.		Amount of amboceptor dilution (0.5 cc. = 2 units), cc.	Amount of sheep corpuscle emulsion (5 per cent. cc.		Hypothetical results.	Readings.
1 . .	0.1	0.5	0.5	0.4	In water-bath at 37° C. for one hour	0.5	0.5	In water-bath at 37° C. for one hour	C.H.[1]	−
2 . .	0.2	0.5	0.5	0.3		0.5	0.5		C.H.	−
3 . .	0.4	0.5	0.5	0.1		0.5	0.5		S.H.	++
4 . .	0.6	0.5	0.5	0		0.5	0.5		N.H.	++++
5 . .	0.8	0.5	0.5	0		0.5	0.5		N.H.	++++
6 . .	1.0	0.5	0.5	0		0.5	0.5		N.H.	++++
Control	2.0	0	0.5	0		0.5	0.5		C.H.	

Interpretation of Results.—It is highly unfortunate that all workers do not employ a technic which has been standardized in its fundamental requirements. At present there are almost as many variations of technic as there are workers. It is incumbent upon the clinician, therefore to know something about the technic which is being employed in work done for him before he attempts to interpret the results, and it behooves the laboratory worker to criticize his technic impartially, to study the possible sources of error, to eliminate them whenever possible, and above all, to so standardize his method so that it will give constant and comparable results. In case of doubtful reaction, it is much fairer to clinician, laboratory worker, and especially to the patient to secure a second or even a third specimen, than to give an erroneous report.

Interpretation of the results should not be attempted by the laboratory worker unless he is able and willing to go into the clinical evidence. It is even more important that the clinician should be well informed as to the significance of the laboratory results and that he should be able to interpret the variations from the expected.

A negative result absolutely does not mean that syphilis can be excluded, as will be seen by a study of the table (page 153). In primary syphilis the reaction does not appear until the human organism is able to develop antibodies. This time varies with different patients, occurring occasionally as early as the third or fourth day after the appearance of the chancre and sometimes not until secondary signs are in evidence. In a lesion which arouses the suspicion of being a chancre, the diagnostic method of choice is the detection of the

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[1] C.H. means complete hemolysis; S.H., slight hemolysis; N.H., no hemolysis.

Treponema pallidum, for treponemata may be found in about 80 per cent. of cases of primary syphilis, while the Wassermann reaction gives positive results in only about 53 per cent. of cases. Even in the secondary stage, when the highest percentage of positive results is obtained, occasional negative results are seen, occurring when the patient is in a debilitated condition, or uses alcohol to excess. In definite tertiary syphilis, a considerable percentage of negative results is reported. This may be accounted for by the diminished resistance on the part of the patient, alcoholism or recent active treatment. Another factor which explains many negative reactions in late cases is the localization of the lesions in portions of the body not especially accessible to the general circulation or the partial walling-off of gummatous deposits.

TABLE SHOWING THE PERCENTAGE OF POSITIVE WASSERMANN REACTIONS OBTAINED BY DIFFERENT WORKERS IN DIFFERENT STAGES OF SYPHILIS.

	Primary.		Secondary.		Tertiary.		Latent.		Tabes dorsalis.		Paresis.		Congenital.	
	Total No.	Per cent. positive.	Total No.	Per cent. positive.	Total No.	Per cent. positive.	Total No.	Per cent. positive.	Total No.	Per cent. positive.	Total No.	Per cent. positive.	Total No.	Per cent. positive.
Boas's collected cases[1] . .	1060	59.0	3526	90.0	1212	84.1	2503	44.3	159	72.0	405	99.2		
Fildes and McIntosh[2] . .	33	93.9	109	99.0	195	97.0	..	..	..	..	..	..	35	100
Craig[3] . . .	908	89.5	1889	96.1	638	87.4	1173	67.3	..	..	..	..	28	89.2
Swift[4] . . .	16	81.0	76	92.0	45	80.0	85	55.0	38	55.0	3	67.0	4	100
Noguchi[5] . .	23	73.9	79	83.7	65	80.0	59	61.0	..	..	..	..	5	80.0

The fallacy of placing sole dependence upon a blood serum Wassermann reaction is most apparent in syphilis of the central nervous system, where blood-serum reactions are often negative while unmistakable evidence of syphilis is found when the spinal fluid is examined.

Not infrequently patients who are under treatment for syphilis have the impression that a single negative Wassermann reaction means that they are cured. This is unfortunate, since a single negative under such circumstances means absolutely nothing.

The possibility of error in interpreting a strongly positive report is not so great. In the temperate zone a strongly positive reaction ordinarily means syphilis. While there are occasional exceptions, they usually occur in cases where there would be small possibility of clinical confusion, such as leprosy, and in the tropics, frambesia.

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[1] Die Wassermannsche Reaktion mit bes. Berücksichtigung ihrer Klinischen Verwertbarkeit, 2d edition, Berlin, 1914. (Quoted in Special Report Series, No. 21, Medical Research Committee, London, 1918, p. 6.)

[2] Brain, London, 1913, xxxvi, 193.

[3] Am. Jour. Med. Sc., 1915, cxlix, 41.

[4] Arch. Int. Med., 1909, iv, 374.

[5] Serum Diagnosis of Syphilis, J. B. Lippincott Co., Philadelphia, 1911.

Faintly or partially positive results, however, should be interpreted with even greater caution, since either may be seen occasionally in tuberculosis and cancer. With indefinite results, repetition of the test is highly advisable. A plus-minus result should never be interpreted as a positive, especially when obtained with cholesterolized antigens.

The Reaction in Treated Cases.—When treatment is started early in the course of spirochetal invasion and pushed intensively, it often produces the prompt reversal of a positive to a negative but with cessation of treatment the test again becomes positive. In cases of longer standing where time has permitted a more generalized invasion of the body, and where the infection is well seated, a positive reaction is often rendered negative with the utmost difficulty, even with the most intensive treatment by arsphenamine and mercury. Indeed in some cases, referred to as "Wassermann fast," a negative seems unobtainable.

The conversion of a positive result to negative under treatment may be regarded as a favorable sign but treatment should not be stopped because of a random negative Wassermann reaction until the history of the case and accumulated clinical and serological experience shows that a prolonged intermission for rest from treatment and for observation is warranted.

Serological Criteria of a Cure in Syphilis.—To pronounce a patient as cured upon serological grounds alone is not good clinical practice. The entire history must be considered, special consideration should be paid to the recency or remoteness of the infection, the promptness with which treatment was started with relation to the primary infection, the amount, duration, and intensity of treatment, the occurrence of so-called "tertiary" lesions and of signs and symptoms pointing to involvement of the central nervous system or of the aorta. Of equal importance is the searching physical examination of the patient with particular reference to mouth and throat, glands, aorta and central nervous system. As a *third method of examination*, the laboratory offers exceptional resources, yielding valuable evidence, possibly unobtainable in any other way. The laboratory side should include three types of examination:

1. Repeated Wassermann reactions upon the blood serum.
2. Provocative Wassermann reactions.
3. Examination of the spinal fluid.

1. *Series of Wassermann Reactions on the Blood.*—When clinical experience shows that it will be safe to permit a prolonged intermission in treatment, this may be stopped and the Wassermann reaction done with the blood serum at intervals of three months. If any test be positive, treatment should be resumed. If all are negative, the provocative test should be made.

2. *Provocative Test.*—It has been shown that in some cases of latent syphilis showing negative results repeatedly, a positive is obtained after the intravenous injection of small doses of arsphenamine.

This may be explained by assuming that the arsenical preparation destroys spirochetes on account of its power of permeation and its consequent ability to be carried to out-of-the-way foci, and that the liberation of toxins leads to the elaboration of syphilitic antibodies, which give a positive result. Whatever the explanation may be, the fact remains that a number of cases which have been treated to the point where repeated negatives have been secured, respond positively after a provocative dose of arsphenamine.

The technic is simple, involving only the intravenous injections of arsphenamine (0.2 to 0.3 gm.) or neo-arsphenamine (0.4 to 0.5 gm.), and securing specimens of blood at two-day intervals for ten days.

3. *Examination of Spinal Fluid.*—It is the general feeling among modern syphilologists that the spinal fluid should be examined, certainly before the patient is regarded as cured, and at other times in the course of infection if indications arise. The technic for obtaining spinal fluid will be given in a later section (page 371). The examination should include cell-count, globulin determination, Lange colloidal gold curve, as well as Wassermann reaction.

Laboratory Diagnosis of Syphilis of the Central Nervous System.—Whatever has been said regarding the folly of excluding syphilis on the strength of a negative Wassermann reaction obtained with blood serum applies with added force to the diagnosis of any form of syphilis of the central nervous system. This is evident from a cursory examination of the following table. The large majority of cases of tabes dorsalis, paresis, and syphilitic meningo-endarteritis give some indication in the spinal fluid which will assist in making a diagnosis, if not a positive Wassermann reaction, an increased cell-count, increased globulin, or specific curve with the Lange colloidal gold test.

TABLE SHOWING RESULTS OF LABORATORY TESTS IN DIFFERENT FORMS OF CEREBROSPINAL SYPHILIS.[1] PERCENTAGE OF POSITIVE RESULTS.

		Blood, cerebrospinal fluid.								
					Wassermann reaction.					
	Number of cases.	Wassermann reaction.	Cell-count per c.mm.	Globulin (Noguchi reaction).	0.5 cc. (1.0 cc.).[2]	0.4 cc. (0.8 cc.).	0.3 cc. (0.6 cc.).	0.2 cc. (0.4 cc.).	0.1 cc. (0.2 cc.).	0.05 cc. (0.1 cc.).
Cerebrospinal syphilis . .	21	62	86	100	94	88	88	81	33	25
Tabes dorsalis .	30	66	97	87	86	83	69	55	23	4

Reactions done with half quantities of all reagents. Amounts of spinal fluid used by authors shown above, and below, in parenthesis, the amount of which this corresponds in system employed by Hauptmann, Nonne and others.

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[1] From Ellis and Swift, Jour. Exper. Med., 1913, xviii, 162.

SUMMARY.—In the *primary stage* the diagnostic value of the Wassermann reaction is limited, increasing in value as the infection progresses, but the diagnostic method of choice is the search for the Spirocheta pallida, which should be performed routinely in all suspicious sores. In the *secondary stage,* the Wassermann reaction is most constant, since the positive results approach 100 per cent. In the so-called *tertiary stage,* with obscure manifestations, visceral or bony-system, the reaction is valuable, especially in differential diagnosis, but only about 80 to 90 per cent. of positive results are obtained. In *central nervous system syphilis,* the blood-serum reaction may be misleading unless judiciously interpreted, since only about 66 per cent. of cases of tabes dorsalis give a positive reaction. In such cases the spinal fluid examination is very much more dependable. In *congenital syphilis* the percentage of positive is in the neighborhood of 90 to 95 per cent. The reaction is of much importance in children with syphilitic parents, because of the long period of latency seen in many cases. It is particularly useful in cases where adoption is contemplated and no history of the parents obtainable. *Treated cases* must be carefully considered on their own merits after complete survey of history, physical findings, and laboratory examinations.

The Gonococcus Complement-fixation Test.—The general theoretical principles underlying the complement-fixation for gonorrhea are the same as those underlying the Wassermann reaction. In practice the antigen is truly specific. The directions which have been given in the description of the Wassermann reaction for preparing and titrating the complement and hemolytic amboceptor and for making the emulsion of sheep cells, together with those regarding preliminary reactions and controls, apply to the gonococcus-fixation tests. In most laboratories where the latter tests are carried out, Wassermann reactions are done also and it is a simple matter to use the same set of reagents.

The Antigen.—A polyvalent antigen should be used, preferably one containing ten of the strains shown by Torrey to be serologically distinct. The gonococcus may be grown upon the medium described by Thomson or upon ascitic or hydrocele-fluid agar. Stock cultures must be transplanted every forty-eight hours, and the cultures must be kept constantly at 37.5° C., since variations in temperature will result in poor growth. To prepare the antigen take stock cultures twenty-four hours old and make cultures on tubes 1 by 6 inches containing Thomson's medium. These tubes should be incubated twenty-four hours and the growth then transferred to wide-mouthed Blake bottles containing the same medium, planting into each Blake bottle the bacteria from one tube, using a sterile cotton swab for inoculating. The Blake bottles are incubated twenty-four hours and the growth is washed off with sterile, neutral distilled water, using from 5 to 10 cc. of water to each bottle, the amount depending upon the growth. The water is not allowed to remain on the agar for more than a few seconds. The suspension is then autolyzed by placing the container in a water-bath, first at 56° C. for one hour and then at 80° C. for one hour. The autolyzed emulsion is filtered through a Buchner filter well packed with paper pulp and then through a neutral, sterile Berkefeld filter of N or V porosity.[1]

After filtration the antigen should be bottled under aseptic precautions and sterilized on three successive days at 56° C. for thirty minutes on each day. It should be kept in the refrigerator. A preservative may be added, 0.1 cc. of a 1:100 dilution of phenol to each cc. of antigen.

Of late, the writer has secured excellent results with the antigen prepared by the Parke-Davis Company, especially with the cold fixation method.

Titration of the Antigen.—The antigen made according to the foregoing directions should be kept in the refrigerator. It is a distilled water extract of gonococci. Just before use, the desired quantity is placed in a test-tube and made isotonic by adding to nine parts of the watery extract one part of sterile 9 per cent. salt solution. The salt solution should not be added until the day when the antigen is to be used, since it interferes with its stability. The extract which has been made isotonic is now diluted by adding to one part of the extract nine parts of sterile normal salt solution.

The commercial antigen referred to is diluted with nine parts of normal salt solution when employed.

A series of test-tubes is set up. Into these are placed respectively 2.0 cc., 1.8 cc., 1.5 cc., 1.2 cc., 1.0 cc., 0.8 cc., 0.6 cc., 0.4 cc., 0.2 cc., and 0.1 cc., of the 1:10 dilution of antigen. With a new and untried antigen, the quantities may have to be increased or diminished and several trials may be necessary to determine the limits of anticomplementary power.

The hemolytic system is adjusted as described in the section on the Wassermann reaction. Complement dilution is added to each tube, the dilution having been made so that 0.5 cc. of the dilution contains 1.5 units of complement, as shown by previous titration of the complement. Normal salt solution is added to each tube when needed to bring the total volume to 1.5 cc. The tubes are subjected to a temperature of 37° C. for one hour or to a temperature of 8 to 12° C. for four to twelve hours, at the end of which time there are added to each tube 0.5 cc. of 5 per cent. suspension of sheep corpuscles and 0.5 cc. of amboceptor dilution, the latter so made up that this quantity contains 2 units.

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[1] Since new Berkefeld filters are often very alkaline, they should be taken apart and boiled in distilled water for five minutes, scrubbed with a small brush in fresh water, and again boiled in distilled water, repeating the process three times. Then the filter may be set up and hot water allowed to stand in it for five minutes, after which neutral distilled water is allowed to run through until the fluid tests neutral with phenolphthalene. All parts may be sterilized in distilled water in the Arnold sterilizer before use. After use the filter should be boiled in distilled water and scrubbed. It may be attached to the filtering flask and sterilized ready for use.

The tubes are again incubated for thirty minutes at 37° C. The tube which shows beginning inhibition of hemolysis is said to contain the anticomplementary unit, and less than one-half of this amount should be used in the test. The antigen should be added to the tubes in this quantity for the reaction. If, for example, the tube in the titer which contained 1.8 cc. was entirely clear while the tube containing 2.0 cc. of the antigen showed some inhibition of hemolysis, we might employ 0.9 cc. of the diluted antigen in the reaction.

Performance of the Reaction.—As a control, there should be at hand either the serum of a rabbit who has been immunized with gonococci or the serum of a patient which has given a gonococcus-fixation test previously. If rabbit serum is used as a control, it should be determined before immunization that the rabbit is not one which gives non-specific complement-fixation in the presence of bacterial extracts. Known normal serum must be used as a control. The reaction may be set up according to the table.

GONOCOCCUS COMPLEMENT–FIXATION TEST.

Tube No.	Patient's serum, cc.	Amount of complement dilution (0.5 cc. = 1.5 un.), cc.	Amount of antigen dilution (0.5 cc. = ½ anti-comp. unit), cc.	Amount of 0.9 per cent. NaCl solution, cc.		Amount of amboceptor dilution (0.5 cc. = 2 units), cc.	Amount of 5 per cent. sheep corpuscle emulsion, cc.		Hypothetical results.	Readings.
	Unknown									
1	0.1	0.5	0.5	0.4	In water-bath at 37° C. for one hour or in ice-chest for four to twelve hours.	0.5	0.5	In water-bath at 37 C. for one hour.	?	?
2	0.2	0.5	0	0.8		0.5	0.5		C.H.[1]	
	Normal									
3	0.1	0.5	0.5	0.4		0.5	0.5		C.H.	Negative.
4	0.2	0.5	0	0.8		0.5	0.5		C.H.	
	Positive									
5	0.1	0.5	0.5	0.4		0.5	0.5		N.H.	Strongly positive.
6	0.2	0.5	0	0.8		0.5	0.5		C.H.	
7	..	0.5	0.5	0.5		0.5	0.5		C.H.	Antigen control.
8	..	0.5	1.0	0		0.5	0.5		C.H.	Antigen control.

Clinical Value of the Gonococcus Complement-fixation Test.—The principal objection to the complement-fixation test for gonorrhea is the difficulty which is encountered in preparing the antigen. This arises from the fact that a number of strains of the gonococcus must be kept alive by frequent transplantation and that the media which are used for this organism are troublesome to prepare.

It is rather generally agreed that the reaction is specific in that positives are not given in any condition except gonococcus infections. The antibody, however, is often not present in large quantities even in definitely gonorrheal infections, because the process may be a limited one. This reduces the possibility of securing positive reactions when infection is present. The reaction is rarely present in the first four to six weeks of a urethritis. Kolmer states that in exacerbations of a chronic urethritis, a positive reaction is secured in about 80 per cent. of the cases, that the reaction is positive in from 30 to 40 per cent. of the cases with mild involvement of the prostate gland complicating chronic urethritis, and that from 50 to 80 per cent. of positive results are secured in cases of chronic urethritis with marked involvement of the prostate gland and epididymitis. A positive reaction is secured in about 80 per cent. of the cases of gonorrheal iritis.

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[1] C.H. means complete hemolysis; N. H., no hemolysis.

A negative result cannot be interpreted as excluding gonorrheal infection, while a positive result is usually taken as pointing to the presence of an active focus. In women the reaction is usually negative until the infection has reached the cervical canal, or Bartholin glands, while with children it is often positive with a severe vulvovaginitis. Nearly 100 per cent. of cases with gonorrheal arthritis show positive complement-fixation tests. It must be remembered that the administration of antigonococcus serum or gonococcus vaccine will always be followed by a positive reaction in a patient who has recently had gonorrhea. Schwartz states, however, that the administration of gonococcal vaccines or sera to a person who has never had gonorrhea will not produce a positive reaction in his blood.

Weakly positive reactions are usually interpreted as pointing to an approaching cure, and if the test is repeated after some weeks, it nearly always becomes negative unless meanwhile vaccines have been given or a new infection acquired.

Clinically, one of the chief uses of the complement-fixation test is in the investigation of completeness of cure in candidates for marriage.

The value of the test is brought out by the fact that in 30 per cent. of cases in which no clinical evidence of disease persists, a positive reaction points to the persistence of an unsuspected focus.

Complement-fixation Test in Tuberculosis.—The complement-fixation test in tuberculosis cannot be said to have an established clinical value. A number of methods have been proposed, the essential differences being in the manner of preparing the antigen, and encouraging reports have been made by the proponents, but unfortunately others have been unable to secure equally good results in employing the same methods. While the test is of distinct interest from a scientific standpoint, the technic will have to be perfected before it can become a generally useful diagnostic measure. The antihuman or antisheep hemolytic system may be used and the strength of the reagents should be balanced by careful titration and by use of preliminary sets of reactions as has been described (see page 147). The following methods have been proposed:

Craig's Method.—The antigen is a modification of Besredka's and is an alcoholic extract of several strains of the tubercle bacillus, grown on a liquid medium composed of alkaline bouillon to which has been added a teaspoonful of egg white and egg yolk for each 250 cc. of bouillon. After growth has proceeded for some time, the bouillon is mixed with an equal quantity of 95 per cent. alcohol and the mixture is shaken in a shaking machine for twelve hours after which it is allowed to stand in the incubator at 37° C. for twenty-four hours. It is then shaken for six hours, again incubated at 37° C., and filtered through very fine filter paper or a Berkefeld filter. The filtrates of various strains should be mixed together and should be tested for anticomplementary power and hemolytic properties. The antigen must be stored in the ice-box.

Miller and Zinsser's Method.—Tubercle bacilli are grown upon suitable solid media. The growth is scraped off. The bacilli may be killed with heat or not as desired. Twenty milligrams of the moist tubercle bacillus mass are weighed out, placed in a conical 15 cc. centrifuge tube with 90 mg. sodium chloride. The paste is ground by hand for one hour with a glass rod. Distilled water is added to the point of isotonicity (*i. e.*, 10 cc. should be added to the quantities given here). The antigen is shaken just before using and the heavier particles are allowed to settle out for a few moments. The resulting emulsion should be titrated for anticomplementary and antigenic properties. This antigen utilizes the endotoxins extracted from the bacilli. The writer has failed to obtain with this antigen the high percentage of positive results reported by Miller and Zinsser.

Petroff's Method.—Petroff advocates the use of more than one antigen, and suggests three different methods of preparation:

Antigen I is polyvalent, and is made from six strains of human bacilli and from one of bovine tubercle bacilli. They are grown on potato broth.

The potato broth is prepared by infusing 500 grams of grated potato in 100 cc. of distilled water over night in the refrigerator. The infusion is then filtered from the pulp through gauze. It is titrated and the reaction is adjusted to 1 per cent. acid, after which it is heated in the autoclave for thirty minutes at a pressure of 15 pounds, and is filtered through filter paper. The clear brown fluid is titrated again, and the reaction adjusted to 0.5 per cent. acid. Glycerin is added to the strength of 4 per cent. and the broth is divided among 250 cc. bottles, placing about 50 cc. in each bottle. It is then sterilized by fractional sterilization in the Arnold sterilizer on three successive days.

After the bacilli have grown in the potato broth for six weeks, the broth is carefully filtered through several layers of filter paper, using suitable care to prevent contamination of the filtrate. Preservatives are not employed, since they eventually render the filtrate anticomplementary. Petroff states that when growth has proceeded sufficiently to produce an antigen of proper strength, the color of the potato broth changes from dark brown to golden yellow. The filtered antigen is placed in small ampoules containing 2 cc. each. These are stored in the refrigerator.

Antigen II is monovalent. Two hundred and fifty milligrams of dried and pulverized tubercle bacilli are extracted with 50 cc. of 1 per cent. aqueous solution sodium hydrate at 38° C. for five to ten days, when the extract is carefully neutralized with decinormal hydrochloric acid. Petroff found that the antigen was quite stable.

Antigen III.—Two hundred and fifty milligrams of dried and pulverized tubercle bacilli are extracted with 50 cc. of Merck's reagent methyl alcohol at 38° C. for five to ten days, when the bottle is corked tightly and set away in a dark place at room temperature. At the end of several weeks' time, the supernatant clear fluid is pipetted off. The antigen is diluted with normal salt before use.

After careful titration of the complement and amboceptor, Petroff recommends the use of the antigen in an amount equal to one-fourth of its anticomplementary unit. He uses an antisheep hemolytic system and allows the incubation of antigen, human serum and complement to proceed for one and a half hours. Presence of native antisheep amboceptor is noted and allowance is made.

Wilson and von Wedel's Method.—These workers employed the reagents in one-tenth the original Wassermann quantities. The antigens are prepared either from a number of stock cultures of human tubercle bacillus or from a strain used for the production of tuberculin, grown in glycerin broth for from three weeks to three months and then killed by heating in the Arnold sterilizer for one hour. The culture is then filtered through filter paper. The filtrate is discarded and the residue placed in absolute alcohol in the proportion of one volume of residue to ten volumes of alcohol. The mixture should be shaken thoroughly by hand and placed in the ice-box for two weeks when it is filtered through paper. The filtrate is discarded and the residue remaining on the filter paper washed with absolute alcohol into a centrifuge tube. After centrifugalization the alcohol is removed and ether is added to the sediment, which is again centrifugalized. The ether is discarded and the centrifuge tube, plugged lightly with cotton, is allowed to stand at room temperature over night so that the residual ether may evaporate. One gm. of powder is emulsified in a large mortar with 200 cc. of normal saline and the emulsion is heated for one hour at 80° C. After standardization, it should be diluted so that the test-dose contains at least 2 antigenic units, and one-fourth or less of the anticomplementary dose. The unit is determined by titrating various amounts of antigen with 0.01 cc. of known positive tuberculous serum. The standard dilution of the two antigens employed by Wilson was 1:50.

Von Wedel has shown that some sera from active tuberculous cases gave negative results when the test was made on the first day after bleeding but reacted positively when the same specimens were kept in the ice-box for a week and were tested later. Sera from known negative cases gave negative results when so tested. Von Wedel also recommends carrying out the test with the quantity of serum usually employed in the Wassermann reaction (0.01 cc. for the system as modified, 0.02 cc., and 0.04 cc., with controls for each containing twice the quantity). Of course the result is considered only when the control tube shows complete hemolysis.

Wilson has shown that the sera obtained from different guinea-pigs varies greatly in its fixability by definite tuberculous serum, and that complement which is quite active in the hemolytic system may not be efficient for tuberculosis complement-fixation. Therefore she recommends testing the serum from each guinea-pig for its fixability with tuberculous antigen and known tuberculous serum before use in diagnostic tests.

Clinical Value of the Complement-fixation Test for Tuberculosis.—At present there is no general agreement as to the technic and there is a great disparity in the results obtained by different workers, the most confusing feature being that no two workers have obtained similar results in using the same methods. The reaction is still in the experimental stage, and much work will have to be done to develop a technically reliable method before the results of observations can be used as a basis for interpreting the significance of future findings. However, it appears that even with the present methods, a definitely positive reaction added to suggestive clinical findings assists in establishing a positive diagnosis, that a negative result does not exclude tuberculosis; and that positive results are more frequent with active than with inactive cases.

From Petroff's method, Brown and Petroff have drawn the following conclusions:

Far advanced cases react positively more frequently than do the moderately advanced, and the moderately advanced than the incipient. All patients with tubercle bacilli do not react positively (about 10 per cent. are negative). The complement-fixation test and the intradermic tuberculin test do not run a parallel course, the latter being far more persistent. A positive fixation text suggests observation of the patient for a time. The complement-fixation test is of more value to the clinician when negative than when positive in the determination of what patients need treatment.

Von Wedel states that his results indicate that 100 per cent. of nontubercular cases will give absolutely negative results; that nearly 100 per cent. of primary and active cases will give positive results with the exception of the dying cases, and that about 25 per cent. of the partially inactive and the active cases will give only weakly positive results.

CHEMICAL EXAMINATION OF THE BLOOD.

Introductory.—Among its many functions, blood serves as a medium which carries food substances from the gastro-intestinal tract to the tissues and carries waste products from the tissues to the different organs by which they are secreted from the body. It is evident that the chemical examination of the blood would yield information of diagnostic value; much more, indeed, than may be obtained by the mere analysis of any of the excretions alone. The study of the blood gives us a very accurate idea as to the increase of certain unconsumed food substances, such as sugar, and the accumulation of undue amounts of waste materials, so that an insight may be had into the actual excretory capacity of the organism. The substances for which the present methods are particularly adapted are: blood sugar, the non-protein nitrogen of the blood, blood urea, blood creatinine, uric acid, and blood chlorides.

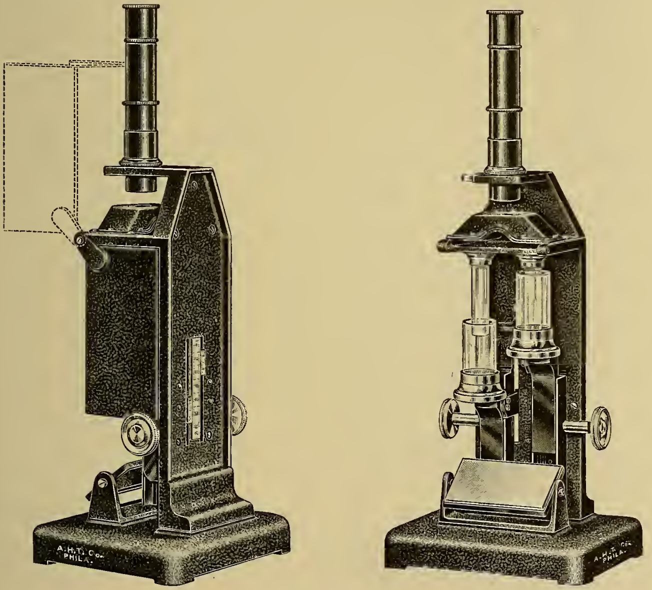
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Fig. 48 Fig. 49

Figs. 48 and 49.—Duboscq colorimeter.

The development of microchemical colorimetric methods by Folin, by Benedict, and by Myers has placed within reach of the clinical laboratory quantitative chemical methods for the determination of many of the constituents of the blood. These methods have been reduced to sufficiently simple terms so that they may be employed by any one who has been trained in quantitative analysis. The chief difficulties are those attendant upon securing chemically pure reagents and upon making up the standard solutions with extreme accuracy. Once they have been surmounted, the actual performance of the determinations need be no more difficult than the determination of the amount of chlorides in the urine, and will certainly reveal information of great value.

The most costly piece of apparatus will be the colorimeter. The high price of the Duboscq instrument (Figs. 48 and 49) probably deterred the more prompt general employment of colorimetric methods. While this apparatus is excellent it is fortunately not the only one now available on the market. Kober has devised a very good apparatus which is made in this country, is somewhat less expensive than the Duboscq instrument and has certain points of mechanical superiority (Fig. 50). Bock and Benedict have brought out a colorimeter (Fig. 51) which some workers prefer to any of the larger models, feeling that when the colors are properly matched the two halves of the optical field blend more perfectly than with other models. Like the Duboscq and the Kober, it employs the plunger principle. It is extremely simple, light, and even less expensive than the Kober. (Manufactured by the Klett Mfg. Co., Inc., New York City). It is the one which the writer

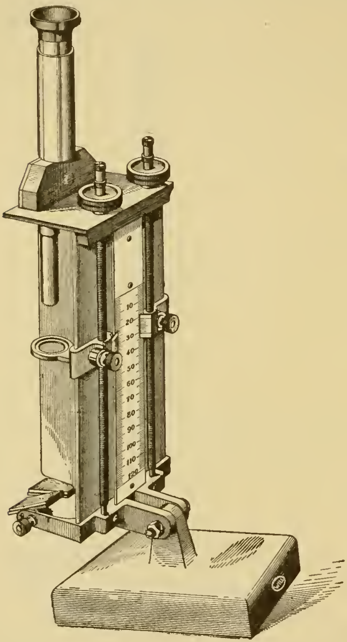

Fig. 50.—Kober colorimeter.

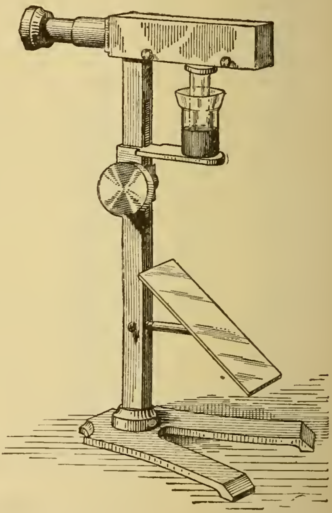

Fig. 51.—Bock-Benedict colorimeter.

employs for routine work because of the superior matching of the color fields. Myers suggests a very inexpensive outfit, an adaptation of the principle used in the hemoglobinometers of the Gower type (Figs. 52 and 53) in which a given quantity of standard solution is placed in one tube and the unknown is placed in the other, a suitable diluent being added to the unknown until the colors in the contents of the two tubes have the same depth of color.

In the Duboscq, Kober, or Bock-Benedict instruments, the cups are placed in position and the colorimeter is set on a bench in front of a good light, preferably from the north. The mirrors are than adjusted so that both halves of the visual field are illuminated equally. The standard solution is placed in the left hand cup. In the first two forms, the cup must be brought to the zero mark to make certain that no air-bubbles are left in the cup to interfere with the passage of the light rays. With the cup raised as far as possible, the position of the zero mark of the vernier scale with regard to the other scale should be noticed. If they do not correspond, the vernier should be adjusted until the two zero marks are at exactly the same level. This may be done readily with the Kober, but is impossible with the Duboscq, where the necessary correction must be noted and allowance made in the readings. The cup containing the standard is then lowered into the desired position, usually so that the reading is at ten, fifteen, or twenty mm. and the right hand cup may be filled with the unknown. This is manipulated as was the other cup, raising it to the zero mark to expel the air, and correcting for the zero mark in the same way. In using the Bock-Benedict instrument, the cup for the standard requires no

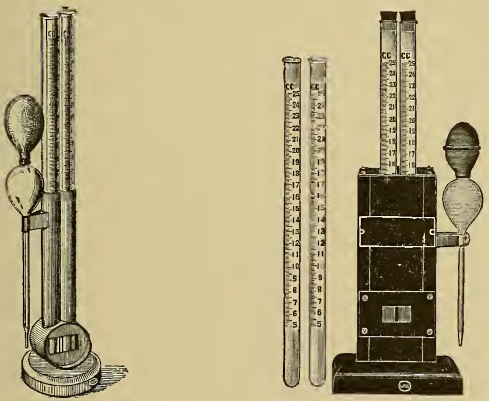

Fig. 52 Fig. 53

Figs. 52 and 53.—Myers' colorimeter.

adjustment after filling; the cup containing the unknown is filled as with the other instruments.

When the right hand cup has been filled with the unknown and adjusted properly, it is lowered gradually by the observer while he keeps his eye at the ocular of the instrument. The depth of color in one side of the optical field will remain stationary, of course, while that in the other grows increasingly deeper. When the two halves match, the reading is taken. It is desirable to take three or four readings with the same unknown and then average the results.

Folin and Denis emphasize certain details in the use of the colorimeter. The zero points should be properly adjusted. The optical parts of the instrument should be thoroughly freed from dust so that hardly a single black speck is visible in either field. Care should be taken that the instrument is optically correct and that the two fields are equally lighted. The instrument should be used in a comfortable position and in a suitable light. Folin recommends putting the instrument on a stool about the height of an ordinary chair in the middle of the laboratory several feet from the window, and sitting at the side of the instrument instead of directly behind it facing the scale. In reading an unknown, both cups should be filled with the standard which should be read against itself to become familiar with the appearance of the standard solution. The standard in the right hand cup is then replaced with the unknown. Folin recommends making only one very careful reading of the unknown.

The principle of the colorimeter is that that quantity of the substance in solution may be measured by the depth of color change produced. The depth of color change is then directly proportional to the strength of the solution and inversely proportional to the height of the column in the colorimeter cup. The equation which is used in calculating the results of colorimeteric determination is:

$$\mathrm{RS} : \mathrm{RX} = \mathrm{CX} : \mathrm{CS},$$

in which RS is the reading of the standard, RX, the reading of the unknown, CX represents the concentration of the unknown and CS is the concentration of the standard. The reading of the standard and the concentration of the standard are known factors; when the reading of the unknown is ascertained, these three factors may be substituted in the above equation, and the concentration of the unknown determined. With most of the methods given in this manual which employ colorimetric determination, a short formula will be given.[1]

Routine Procedure.—The tests which will be employed depend upon the nature of the case. *In diabetes,* the determinations which would be desired would include at least a determination of the blood sugar and of the CO_2 combining power according to Van Slyke's method.

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[1] A list of apparatus required for chemical examination of the blood will be found in the appendix. The apparatus required for the tests for acidosis is specified with the descriptions of the tests.

These represent minimum requirements. Blood examination must be accompanied by a careful examination of the urine, with especial reference to the amount excreted in twenty-four hours, the amount of sugar in a twenty-four-hour specimen, the presence of the acetone bodies, and also the presence of albumin and the microscopic examination. To these methods of examination may be added Sellards' bicarbonate tolerance test, and in indicated cases, the determination of the carbon-dioxide tension of the alveolar air. The determination of the diastatic activity of the blood may be helpful.

With nephritis, the determinations of especial value include the estimation of blood urea, creatinine, and uric acid, and the determination of the CO_2 combining power. When carrying out these determinations, the determination of blood sugar involves very little additional labor and often contributes data of value. The determination of non-protein nitrogen adds little data of clinical usefulness when the blood urea has been determined, and imposes much labor. The work upon the blood should be supplemented by a careful study of the urine, particularly with reference to the presence and amount of albumin in a twenty-four-hour specimen, the amount voided in twenty-four hours, the microscopic study of a fresh specimen, the response to the Mosenthal diet, and the excretion of phenolsulphonephthalein.[1]

In gout the determination of urea, creatinine and especially of uric acid should be made. For the normal values and the findings in various diseases, the table on page 168.

Obtaining Specimen of Blood.—The specimen should be obtained twelve to sixteen hours after a meal, preferably in the morning before breakfast, after a night during which no food has been taken. The specimen should be drawn directly from the vein into a tube into which has been placed powdered potassium oxalate. Twenty milligrams of potassium oxalate is sufficient for 10 cc. of blood. An excess should be avoided. If a syringe is used the oxalate may be placed in the barrel of the syringe. Since other tests upon the blood may be performed at the same time, it is desirable to obtain at least 10 or 15 cc. of blood. For obtaining the specimen, a McRae needle or one of the type suggested by the writer, may be used. The technic for venipuncture has been described on page 126.

Choice of Methods.—For chemical determinations of sugar and the nitrogen derivatives, two methods have been given, those devised by Folin and Wu, and those adapted by Myers and his co-workers. The choice is largely a matter of individual preference.

Folin and Wu employ tungstic acid as a protein precipitant. The technic for this preliminary step, which is common to all their methods, is given with the description of the determination of blood sugar, and to save needless repetition, is not repeated in describing the following methods devised by the same authors. When a complete examina-

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[1] An excellent discussion of the chemical changes in the blood in disease is given by V. C. Myers in the Jour. Lab. and Clin. Med., 1920, v, 343, *et. seq.*

SIGNIFICANT CHEMICAL CHANGES IN THE BLOOD IN DISEASE.

	Urea N., mg. to 100.	Sugar, per cent.	CO_2 combining power, cc. to 100.	Creatinine, mg. to 100.	Uric acid, mg. to 100.	Non-protein N, mg. to 100.	Cholesterol, per cent.	Chlorides as NaCl, per cent.	Diastatic activity.
1. Normal	12–15	0.09–0.12	50–75	1–2	2–3	25–30	0.14–0.17	0.57–0.62	15–20
2. Beginning pathological	20	0.15	−45	3.5	4	35	0.19	{ −0.55 +0.65	25
3. Renal diabetes	....	0.08–0.12							
4. Mild diabetes	....	0.15–0.30	....	....	....	....	....	....	25–40
5. Severe diabetes	20	0.30–1.20	50–10	2–4	4–10	....	0.2–0.8	0.5	35–75
6. Gout	....	....	....	....	4–10				
7. Early interstitial nephritis	15–25	0.12–0.15	....	2–3.5	5–12				
8. Acute nephritis	40–100	0.12–0.18	45–20	2–6	5–15				
9. Parenchymatous nephritis	20–50	0.12–0.20	....	2–4	2–5	....	....	to 0.75	
10. Terminal interstitial nephritis	60–300	0.12–0.24	12	5–28	5–27	100–350	to 0.30	to 0.46	20–50
11. Bichloride poisoning	300	0.12–0.20	—	33	15	370	0.35	0.5–0.6	
12. Double polycystic kidney	75	0.2	....	8	5				
13. Prostatic obstruction	12–40	0.11–0.16	....	1.5–3.5	3–9				
14. Acute intestinal obstruction	45–120	....	....	....	....	75–170			
15. Eclampsia	10–25	....	58–43	....	....	25–45			
16. Cholelithiasis	....	....	....	....	....	....	0.13–0.30		
17. Pernicious anemia	....	....	....	....	....	....	to 0.06		

From Myers: Jour. Lab. and Clin. Med., 1920, v, 344.

tion is to be made, an adequate quantity of blood is treated with tungstic acid and portions of the filtrate used for the different determinations.

The same rule has been followed in describing Myers's set of tests, though it should be noted that his method for uric acid is an adaptation of Folin and Wu's and calls for precipitation of the blood by tungstic acid rather than by picric acid, as do his other tests.

General Method of Procedure.—If Folin and Wu's methods are to be used, precipitate 10 cc. of blood with tungstic acid (see page 172) and use portions of the filtrate for determinations of sugar, creatinine, urea, non-protein nitrogen, and uric acid, or as many of these tests as may be required. If Myers's methods are to be followed, precipitate 5 cc. of blood with picric acid (see below) for sugar and creatinine determinations, precipitate another 5 cc. portion with tungstic acid (see page 194) for uric-acid determination, use whole blood for the estimation of urea, and treat a portion with trichloracetic acid for the determination of non-protein nitrogen. While this may seem somewhat more involved, the author has not found the latter set of tests required an appreciably greater number of operations than the former.

DETERMINATION OF BLOOD SUGAR.

Method of Myers and Bailey (Modified from that of Benedict and Lewis).—*Principle.*—The blood proteins are precipitated by picric acid in saturated solution. The addition of sodium carbonate to picric acid solution results in the formation of sodium picrate, which in the presence of heat, is reduced by the sugar in the filtrate with a color change which may be determined by colorimeteric methods, using as a standard a solution of glucose of known strength.

Reagents Required.—1. Standard solution of glucose, 0.02 per cent. in a saturated solution of picric acid.

2. Saturated solution of sodium carbonate (about 22 per cent.).

3. Picric acid, pure, crystals.

For method of testing purity of picric acid and for purifying it, see page 442.

Procedure.—Should only the blood sugar determination be desired, 2 cc. of blood are laked by the addition of 8 cc. of water. If, however, one desires to determine the amount of creatinine at the same time, 5 cc. of blood may be laked by the addition of 20 cc. of water. This laking is accomplished conveniently in a 50 cc. round-bottomed centrifuge tube. Dry chemically pure picric acid is added to the point of saturation. The picric acid is stirred thoroughly with a glass rod and is added bit by bit until a few undissolved crystals are seen at the bottom of the tube. After the picric acid has been thoroughly mixed with the laked blood, the mixture is allowed to stand about five minutes when separation may be hastened by centrifugalization, and the supernatant fluid is filtered through paper. If a heavy centrifuge be not available, it may, of course, be filtered without centrifugalization. The precipitate is composed largely of protein, sugar, of course, being

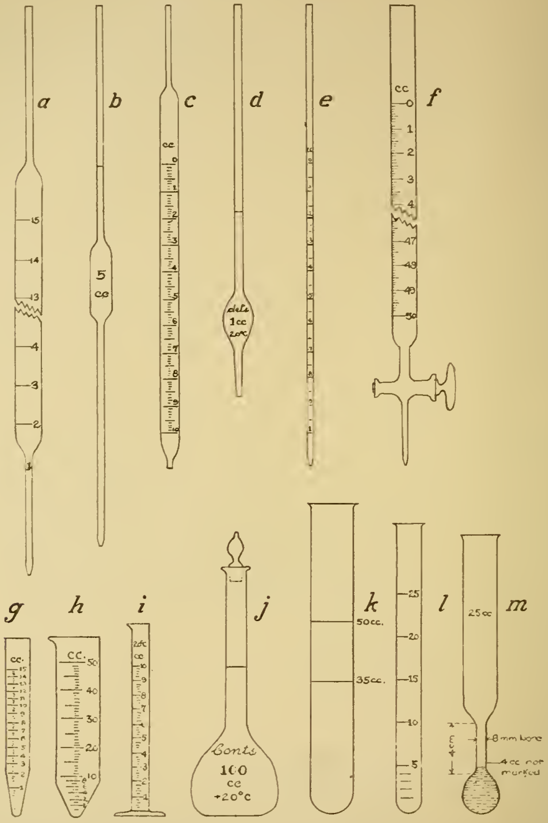

FIG. 54—Types of glassware used in blood chemistry. *a*, Folin pipette; *b*, volumetric pipette with one mark only; *c*, 10 cc. pipette, graduated in 0.1 cc.; *d*, Ostwald pipette; *e*, 1 cc. pipette, graduated in 0.1 cc.; *f*, 50 cc. burette, graduated in 0.1 cc.; *g*, graduated 15 cc. centrifuge tube; *h*, graduated 50 cc. centrifuge tube; *i*, 10 cc. cylindrical graduate; *j*, volumetric flasks (various capacities); *k*, Folin ignition tube; *l*, Myers sugar tube; *m*, Folin sugar tube.

in solution. Three cubic centimeters of the filtrate are placed in a special Myers' sugar tube (Fig. 54, *l*). At the same time one should set up as a control a tube of the same type containing 3 cc. of the standard 0.02 per cent. glucose solution made in saturated picric acid solution. To the tube containing the unknown and also the control tube there should be added 1 cc. of sodium carbonate solution. The two tubes are then placed in a beaker of water, which is boiled for at least ten minutes. At the end of this time the fluid in the tube containing the standard solution is brought to the 10 cc. mark with distilled water. The fluid in the tube containing the unknown is brought to the 10, 15, 20, or even 25 cc. mark as may be required to bring the colors to about the same depth. When the colors have been matched roughly in this way, the standard solution is brought to the left hand cup of the colorimeter and the fluid from the tube containing the unknown is placed in the right hand cup of the colorimeter. The standard solution is placed at the 10 mm. mark and the tube containing the unknown is moved back and forth until the two fields match when the reading of the unknown is taken.

Résumé of Method.—

1. To 2 cc. of oxalated blood add 8 cc. of distilled H_2O.
2. Add dry picric acid to saturation, mixing thoroughly.
3. Let stand five minutes, then centrifugalize.
4. Filter and employ filtrate as shown below (tube with unknown).
5. Place 3 cc. standard 0.02 per cent. glucose solution in Myers tube.

TUBE WITH STANDARD.	TUBE WITH UNKNOWN.
6. Put in 3 cc. of standard glucose solution.	Put in 3 cc. of filtrate.
7. Add 1 cc. sat. sol. Na_2CO_3.	Add 1 cc. sat. sol. Na_2CO_3.
8. Stand in beaker of boiling water 10 minutes.	Stand in beaker of boiling water 10 minutes.
9. Add distilled H_2O to 10 cc.	Add distilled water to 10, 15, 20 cc. mark, as needed to approximate color of standard.

10. Compare tubes in colorimeter.

Calculation.—When the procedure has been carried out as outlined above, the following formula may be used for computation:

$$D \times \frac{0.1}{R} = \text{the blood sugar in per cent.}$$

Folin and Wu's Method for Sugar Determination.—Folin has criticized the picrate methods stating that in his hands they have given results which are higher than with his method. He suggests that the picrate methods may be subject to sources of error similar to those encountered in his original method for blood creatinine. The color developed with this method is far more intense than that developed by the alkaline picrate so that a small fraction of a milligram of dextrose is all that is required for a determination. While sensitive to traces of dextrose, the copper solution is not affected by creatinine or uric acid in quantities corresponding to 50 mg. per 100 cc. of blood.

Principle.—The proteins are precipitated from the blood by tungstic acid. The protein-free filtrate is allowed to reduce a weakly alkaline solution of copper tartrate, with the aid of heat. Folin and Wu's molybdate phosphate reagent is added, which forms an intense blue color in the presence of cuprous oxide in acid solution. The amount of dextrose may be estimated colorimetrically by comparison with a standard containing a known amount of dextrose which has been subjected to similar treatment.

Reagents Required.—1. *Stock and Standard Sugar Solutions.*—The stock sugar solution is prepared by dissolving 1 gm. pure anhydrous dextrose in water and diluting to 100 cc.

When pure dextrose is not available, a standard solution may be made from invert sugar prepared from cane sugar. Exactly 1 gm. cane sugar is placed in a 100 cc. volumetric flask (Fig. 54, *j*) with 20 cc. of normal hydrochloric acid. The mixture is allowed to stand at room temperature overnight. This step may be carried out in ten minutes by rotating the flask and its contents continuously in a water-bath at 70° C. The acid is neutralized by adding 1.68 gm. sodium bicarbonate and about 0.2 gm. sodium acetate. It is necessary to shake for a few minutes to remove the carbonic acid. The flask is filled to the 100 cc. mark with distilled water. An additional 5 cc. of water is added, since 1 gm. cane sugar yields 1.05 gm. invert sugar. The contents of the flask are mixed thoroughly and poured into a bottle with a few drops of xylene or toluene. The stock solution, made either from dextrose or invert sugar, keeps indefinitely.

Two standard solutions are prepared from the stock solution, one by diluting 5 cc. of the stock solution to 500 cc. with distilled water and the other by diluting 5 cc. of the stock solution to 250 cc. Two cc. of the first solution contains 0.2 mg. dextrose, and 2 cc. of the second contains 0.4 mg. The standard solution may be kept with xylene or toluene, but should not be depended upon for more than a month.

2. *Alkaline Copper Tartrate Solution.*—Forty gm. anhydrous sodium carbonate are dissolved in 400 cc. distilled water and transferred to a liter volumetric flask. In this solution are dissolved first 7.5 gm. tartaric acid and then 4.5 gm. crystallized copper sulphate. The volume is made up to 1 liter after thorough mixing. Should a sediment appear as the result of impure chemicals, the clear supernatant fluid may be removed with a siphon.

3. *Folin and Wu's Molybdate-phosphate Reagent.*—In a liter beaker is placed 35 gm. molybdic acid, 5 gm. sodium tungstate, 200 cc. of 10 per cent. sodium hydrate and 200 cc. of water. This is boiled vigorously for twenty to forty minutes to remove nearly all the ammonia which the molybdic acid contains as an impurity. The solution is cooled and diluted to 350 cc. when 125 cc. concentrated phosphoric acid (85 per cent.) are added and the volume brought to 500 cc. with distilled water.

4. *Sodium Tungstate.*—A 10 per cent. solution in distilled water. Folin and Wu have found a number of brands satisfactory.. (It may be obtained from the American Vanadium Corporation, Primos, Pa., who are successors to the Primos Chemical Co., mentioned by Folin and Wu as having made this chemical for them, and market a brand known as "Dr. Folin's quality.") The impurity which may be bothersome is an excess of carbonates. This may be tested for as follows (Folin and Wu): A drop of phenolphthalein is added to 10 cc. of the 10 per cent. sodium tungstate solution, and $\frac{N}{10}$ HCl is added from a burette until the color fades. Each cc. of the decinormal acid represents 1.06 per cent. of sodium carbonate. With a satisfactory preparation, the amount of acid required for neutralization should not exceed 0.4 cc.

5. *Two-thirds Normal Sulphuric Acid Solution.*

Procedure.—Blood is obtained by venipuncture and oxalated as has been directly previously (see page 167). When only a sugar examination is desired, start with 2 cc. of blood, but if a complete set of tests be required, 10 cc. of blood should be used and the reagents added in corresponding proportions.

The blood is placed in a flask having a capacity fifteen to twenty times that of the volume taken. To it should be added in turn, seven times its volume of water, 1 volume of 10 per cent. sodium tungstate, and 1 volume of two-thirds normal sulphuric acid; the final dilution of blood is 1 to 10. The contents of the flask should be mixed after the addition of each reagent and shaken constantly during the addition of the sulphuric acid. Then the mouth of the flask is closed with a rubber stopper and the flask is given a few vigorous shakes. When conditions are right, scarcely an air-bubble is formed.

When properly coagulated, the color of the coagulum changes from pink to dark brown. When the change does not occur, the fault lies in the addition of too much oxalate. This fault may be remedied by adding 5 per cent. sulphuric acid, drop by drop, shaking vigorously after each addition, and allowing the flask to stand for a few minutes before adding more, until coagulation is complete. The mixture should be poured on a filter large enough to hold the entire contents of the flask, when the funnel is covered with a watch glass to prevent evaporation. If the filtrate be cloudy, the first few cubic centimeters should be returned to the funnel.

A water-bath is heated to boiling. Into one of the special Folin sugar tubes (Fig. 54) graduated to 25 cc. is placed 2 cc. of the blood filtrate. Into each of 2 similar tubes are placed 2 cc. of the standard sugar solutions, containing respectively 0.2 and 0.4 mg. of dextrose. To each tube is added 2 cc. alkaline copper tartrate solution. The surface of the mixture should reach the constricted part of the bulb. If the bulb be too large, 0.5 cc. or less of diluted (1:1) alkaline copper tartrate solution may be added. If this does not suffice to bring the contents to the narrow part, or if the tube is so small that the fluid does not come up to the neck, the tube should be discarded. The tubes are placed in boiling water and heated for six minutes. They are removed and cooled in a cold water-bath for two to three minutes without shaking, when 2 cc. of molybdate-phosphate solution are added to each tube. After the cuprous oxide has dissolved, the contents of all tubes are diluted to the 25 cc. mark. Rubber stoppers are inserted and the solutions are mixed. The unknown solution is compared colorimetrically with that standard whose color intensity most nearly approximates its own.

Résumé of Method.—

1. Place 1 volume of oxalated blood in an Erlenmeyer flask.
2. Add (*a*) 7 volumes of distilled water.
    (*b*) 1 volume of 10 per cent. sodium tungsate.
    (*c*) 1 volume of two-third normal sulphuric acid.
   Rotate flask constantly while making additions.
3. Filter when coagulation is complete. Employ portion of filtrate as shown below.
   (Steps 1 to 3 inclusive are the same as those employed for obtaining protein-free filtrate for other determinations by Folin and Wu's methods. If these be desired, take a larger quantity of blood and increase quantities of reagents in 2 accordingly.)

	TUBE WITH FIRST STANDARD.	TUBE WITH SECOND STANDARD.	TUBE WITH UNKNOWN.
4.	2 cc. standard sugar solution (contains 0.2 mg. dextrose).	2 cc. standard sugar solution (contains 0.4 mg. dextrose).	2 cc. protein-free filtrate.
5.	Add 2 cc. alkaline copper tartrate solution.	Add 2 cc. alkaline copper tartrate solution.	Add 2 cc. alkaline copper tartrate solution.
6.	Place in boiling water 6 minutes.	Place in boiling water 6 minutes.	Place in boiling water 6 minutes.
7.	Remove and place in cold water 2–3 minutes.	Remove and place in cold water 2–3 minutes.	Remove and place in cold water 2–3 minutes.
8.	Add 2 cc. molybdate-phosphate solution.	Add 2 cc. molybdate-phosphate solution.	Add 2 cc. molybdate-phosphate solution.
9.	Allow cuprous oxide to dissolve.	Allow cuprous oxide to dissolve.	Allow cuprous oxide to dissolve.
10.	Dilute to 25 cc. mark with water.	Dilute to 25 cc. mark with water.	Dilute to 25 cc. mark with water.

Compare contents in colorimeter.

Calculation.—The calculation may be made by this formula, S representing the reading of the cup containing the standard and R that containing the unknown.

$$\text{Mg. of sugar per 100 cc. of blood} = \frac{S \times 100}{R}$$

Findings.—In normal cases the findings run between 0.09 and 0.12 per cent., according to the figures of Lewis and Benedict, and Myers and Bailey. Myers and Bailey, however, state that there are a number of hospital cases which show blood sugars as high as 0.14 per cent. In renal diabetes there is no increase in the amount of sugar in the blood. An increased amount of blood sugar has been reported in fatal cases of mercuric bichloride poisoning with nephritis; in a case of chronic interstitial nephritis; and in numerous cases of chronic parenchymatous nephritis.

Diastatic Activity of the Blood.—While it has been recognized since the time of Magendie that the blood possessed active diastatic properties, there had been no conclusive work done in regard to this property in human beings subject to diabetes till the work of Myers and Killian. From their study they conclude that the diastatic activity of the blood is increased in cases of diabetes and also in cases of nephritis. The method is simple.

Reagents Required.—Those specified for blood and sugar determinations with the addition of 1 per cent. solution of soluble starch in water. The sugar content of this should be determined.

Procedure.—Oxalated blood is obtained as previously directed. Two tubes are prepared, each containing 2 cc. of blood. One tube serves as a control. This should be marked C. The other tube is for the purpose of determination. This should be marked A. To the tube marked C should be added 8 cc. of water, while to the tube marked A are added 7 cc. of water. These tubes are placed in an incubator or water-bath at 40° C. for ten minutes, at the end of which time 1 cc. of a 1 per cent. solution of soluble starch is added to tube A. The tubes are again placed in the incubator or water-bath at 40° C. for fifteen minutes, at the end of which time the sugar is determined from both tubes according to the method previously given of Myers and Bailey. The amount of sugar in the starch solution should have been determined and should be expressed in milligrams.

Résumé of Method.—

	TUBE A.	TUBE C.
1.	2 cc. of oxalated blood.	2 cc. of oxalated blood.
2.	Add 7 cc. of distilled water.	Add 8 cc. of distilled water.
3.	Leave in water-bath at 40° C. for ten minutes.	Leave in water-bath at 40° C. for ten minutes.
4.	Add 1 cc. of 1 per cent. solution soluble starch.	
5.	Replace in water-bath at 40° C. and leave fifteen minutes.	Replace in water-bath at 40° C. and leave fifteen minutes.

6. Determine the amount of sugar in each specimen by method given previously (see page 169).

Calculation.—After the amount of sugar in tubes A and C has been determined in percentages according to the Myers and Bailey method, it should be translated to terms of mg. per cc. The number of mg.

in 2 cc. of the blood before starch has been added, as determined by tube C, should be subtracted from the amount of sugar in the specimen to which starch has been added as determined by tube A. From this should be subtracted the corrections for the amount of free sugar in the starch solution. The remainder will represent mg. of the sugar which has been formed from the starch by the diastatic activity of 2 cc. of blood. This may be transferred to terms of percentage since the amount of the starch, being a 1 per cent. solution, contained 10 mg. of starch. For example: If 2 cc. showed that there was 0.22 per cent. of sugar in the blood, 2 cc. of blood would contain 4.4 mg. If tube A showed that the conversion of starch by the diastase had brought the total of blood sugar plus converted starch to 0.4 per cent. sugar, then in 2 cc. there would be 8 mg. of sugar. If the starch solution contained 0.6 mg. of sugar (and it should not exceed this figure) we would subtract 4.4 and 0.6 from 8 leaving a remainder of 3 mg. which represents the diastatic activity of 2 cc. of blood. In other words, the blood was able to convert 30 per cent. of the amount of starch present.

Significance of Findings.—By this method Myers and Killian found that the diastatic activity of the blood in normal subjects ranged from 15 to 20. In cases of diabetes they found the figure ranged from 25 to 75, and in cases of nephritis, from 20 to 50. They thought that the results suggested that increased diastatic activity in both diabetes and nephritis may be an important factor in the production of hyperglycemia; that a fall in the blood diastase would appear to afford a more reliable guide to the efficacy of treatment in diabetes than either the blood sugar or the urinary sugar; and that an increase in the blood diastase may constitute a very early sign of impending diabetes.

Determination of Sugar Tolerance.—Hammann and Hirschman proposed utilizing the determination of alimentary hyperglycemia and glycosuria as a clinical method for sugar tolerance. They found that the maximum concentration reached from 0.1 per cent. to 0.13 per cent. after giving 100 grams of glucose, and that the high point was attained from twenty minutes to one and a half hours.

Their method was essentially as follows: After a night's fast, the patient is given 100 gm. of glucose dissolved in 300 cc. of water, flavored with orange or lemon juice. Blood is taken by venipuncture before giving the glucose, one-half hour later, one hour later, and finally two hours later, and the blood sugar is determined by one of the preceding methods. At the same time-intervals, specimens of urine are obtained and examined for sugar by Benedict's qualitative and quantitative methods.

Hammann and Hirschman found that patients with lowered carbohydrate tolerance presented more pronounced and more prolonged hyperglycemia than was seen in normal individuals, the blood sugar usually exceeding 0.2 per cent. and the reaction lasting from three to five hours. Increased carbohydrate tolerance was indicated by subnormal reaction, the blood sugar showing only an insignificant rise. As far as could be determined from this alimentary test, the disturbance in glucose utilization was essentially the same in diabetes and in other conditions with low sugar tolerance, such as deranged thyroid and hypophysial function, and in nephritis.

It was shown that the normal *renal threshold* for glucose was between 0.17 and 0.18 per cent., that is, that glucose appeared in the urine when the blood sugar reached this concentration.

A normal threshold was found in patients with diminished carbohydrate tolerance, while some otherwise normal individuals have a low renal threshold. Although the threshold is usually normal in mild cases of diabetes, it may be lowered in severe case, this factor contributing to the severity of the condition.

The test proves particularly valuable in the investigation of patients with slight or occasional glycosuria, since it decides whether the glycosuria be due to a metabolic disturbance, to a low renal threshold, or to a combination of both factors. A high renal threshold suggests a more serious disturbance of glucose utilization than would be indicated by a mild glycosuria.

BLOOD UREA.

Method of Myers (Modified from those of Marshall and of Van Slyke and Cullen).—*Principle.*—By the aid of the ferment, urease, the blood urea is converted to ammonium carbonate. This is broken up and the ammonia liberated by the action of a strong alkali (saturated solution of sodium carbonate). By means of a strong current of air, the ammonia is removed and neutralized by sulphuric acid. By Nesslerizing the resultant solution of ammonium sulphate, the amount of nitrogen may be determined.

Reagents Required.—1. Urease, 5 per cent. solution in water, freshly prepared. The brand of urease sold by the Arlington Chemical Company is preferable. It contains monobasic and dibasic phosphates in proper proportion to give a neutral solution when dissolved.[1]

2. Decinormal sulphuric acid.
3. Sodium carbonate, saturated solution.
4. Nessler's solution (Benedict-Bock formula).

This is prepared as follows:

Mercuric iodide	50 gm.
Potassium iodide	35 "
Sodium hydrate	50 "
Distilled water	500 cc.

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[1] Folin and Wu recently called attention to the fact that preparations of urease do not show constant keeping power and suggest the following method of preparation: Transfer to a 200 cc. flask about 3 gm. of permutit powder. Wash this by decantation, once with 2 per cent. acetic acid, then twice with water. Add to the moist permutit in the flask 100 cc. of 30 per cent. alcohol (35 cc. of 95 per cent. alcohol mixed with 70 cc. of water). Then introduce 5 gm. jack bean meal (may be obtained from the Arlington Chemical Co.) and shake for 10 minutes. Filter and collect the filtrate in three or four different small clean bottles. Set one aside for immediate use; it will remain serviceable for at least 1 week at ordinary room temperature, if not exposed to direct sunlight. Put the others on ice where they will remain good for 3 to 5 weeks.

The mercuric iodide is rubbed to a smooth paste in a mortar with a little water and is transferred to a 500 cc. measuring flask. The potassium iodide is ground to a powder in the same mortar and added to the iodide paste in the measuring flask, washing it with about 200 cc. of water. The sodium hydrate is dissolved in about 250 cc. of water and, after cooling, is added with constant shaking to the solution of mercuric iodide and potassium iodide in the measuring flask. The volume is brought to 500 cc. with distilled water. After standing at room temperature until the yellowish-white precipitate, which may settle out at first, has dissolved, the supernatant clear fluid of siphoned off from the dark-brownish-red precipitate.

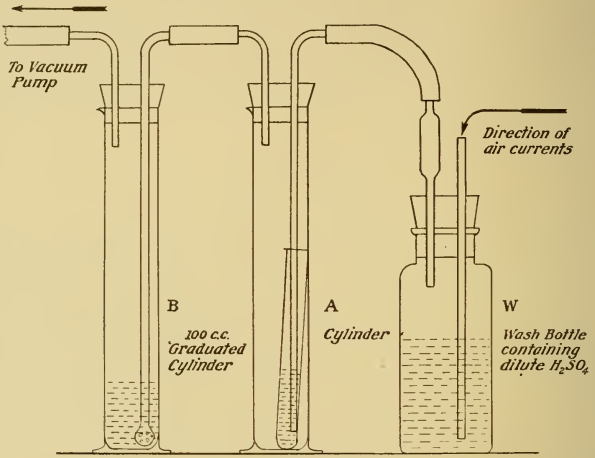
To Vacuum Pump
Direction of air currents
B
100 c.c. Graduated Cylinder
A
Cylinder
W
Wash Bottle containing dilute H_2SO_4

Fig. 55.—Aërating apparatus.

5. A standard solution of ammonium sulphate (containing 1 mg. is nitrogen to 5 cc. of solution). A standard solution to contain 1 mg. of nitrogen per 5 cc. of solution may be prepared by dissolving in a liter of distilled water 0.944 gm. of ammonium sulphate or 0.764 gm. of ammonium chloride, highest purity. Folin states that pure ammonium sulphate may be prepared by decomposing a high grade ammonium salt with sodium hydroxide and passing the ammonium gas which is liberated by this decomposition through pure sulphuric acid with the aid of an air current. The salt, which is redissolved in water, is again precipitated with alcohol, and is finally dried in a desiccator over sulphuric acid.

Procedure.—Blood is obtained by venipuncture and oxalated as previously directed (see page 167). The aërating apparatus is set up

(Fig. 55). It should be tested out before making the determination by connecting it and then turning on the air current.

If all joints are air-tight, air should bubble through the dilute acid solution in wash bottle (*W*). In graduate *B* is placed 2 cc. of $\frac{N}{10}$ H_2SO_4 and 15 cc. of water.

Into a test-tube is placed 2 cc. of the oxalated blood, and to it is added 1 cc. of the 5 per cent. urease solution. The test-tube is then placed in the water-bath at 50° C. for fifteen minutes, at the end of which time the test-tube is placed inside the graduate (*A*) after adding 1 cc. of amyl alcohol to prevent bubbling. With the tube in place and the apparatus ready to connect, 4 to 5 cc. of saturated solution of sodium carbonate is run in with a pipette. The alkali facilitates breaking up of the ammonium carbonate to ammonia. Aëration is carried out by using a strong current of air. The apparatus should be set up as is shown in Fig. 55. The ammonia gas which is freed by aëration is drawn into the second graduate (*A*). Aëration is continued from thirty to forty-five minutes.

The standard should be made up in a 100 cc. graduated cylinder in which are placed 5 cc. the standard ammonium sulphate solution (containing 1 mg. nitrogen), 20 cc. distilled water, 20 cc. 20 per cent. Nessler's solution, and water sufficient to bring the total volume up to 100 cc. To the cylinder which contained the decinormal acid and into which the ammonia has been drawn by the air current is added enough 20 per cent. Nessler's solution to secure maximum color change (this may be 20 cc. or less) and water to make the intensity of the color of the standard and the unknown approximately the same (usually stopping at 30, 50, or 100 cc., for the sake of convenience in computation).

The left hand cup of the colorimeter is then charged with a portion of the standard solution and the right hand cup is charged with a portion of the unknown. The left hand cup is set at 10 mm. and the right hand cup is moved up and down until the color in the two halves of the colorimeter field matches when the reading is taken.

Résumé of Method.—

1. Place 2 cc. of oxalated blood in test-tube.
2. Add 1 cc. of 5 per cent. urease solution.
3. Leave in water-bath at 50° C. for fifteen minutes.
4. Add 1 cc. of amyl alcohol.
5. Place test-tube inside cylinder *A* of aërating outfit.
6. In receiving cylinder *B*, place 2 cc. of $\frac{N}{10}$ H_2SO_4 and 15 cc. distilled water.
7. Add 5 cc. of saturated solution of sodium carbonate to tube in cylinder *A*.
8. Stopper cylinders *A* and *B*, connect up apparatus, and start air current.
9. Allow aëration to proceed for from thirty to forty-five minutes.
10. Take cylindrical graduate *C* of the same size as *B* and mix in it the standard solution.

Treat the contents of the cylinders B and C as follows:

	CYLINDER B (CONTAINS THE UNKNOWN).	CYLINDER C (CONTAINS THE STANDARD).
11.	Contains substances placed in it in step 6 plus the liberated ammonia.	Place in cylinder 5 cc. standard ammonium sulphate solution (contains 1 mg. nitrogen).
12.		Add 20 cc. distilled water.
13.	Add 20 per cent. dilution Nessler's solution to maximum color.	Add 20 cc. of 20 per cent. dilution of Nessler's solution.
14.	Dilute to 30, 50, or 100 cc. to match roughly the color of C.	Bring total volume to 100 cc. with distilled water.
15.	Compare contents of cylinders in colorimeter.	

Calculation.—When the standard cup of the colorimeter is set at 10 mm., divide one-half of the dilution of the unknown by the reading of the unknown and multiply the quotient by 10. This represents the mg. of urea nitrogen per 100 cc. of blood. The result is in terms of nitrogen. If it be desired to convert the urea nitrogen to terms of urea this may be done by multiplying by the factor 2.14.

Method of Folin and Wu.—*Principle.*—The principle is essentially the same as in the foregoing method, except that protein is precipitated by tungstic acid.

Reagents Required.—1. *Sodium Tungstate* (10 per cent.).—See page 173.

2. *Two-thirds Normal Sulphuric Acid.*

3. *Urease Solution.*—See footnote, page 177.

4. *Pyrophosphate Solution.*—Prepared by dissolving 140 gm. of sodium pyrophosphate (U. S. P.) in water, adding 20 gm. of glacial phosphoric acid, and bringing total volume to 1 liter with water.

5. *Standard Nitrogen Solution.*—This may be prepared by dissolving 0.2832 gm. of ammonium sulphate (highest purity) in distilled water and bringing volume to 1 liter with distilled water; 5 cc. of this will contain 0.3 mg. of nitrogen.

6. *Twentieth-normal Hydrochloric Acid Solution.*

7. *Saturated Solution of Borax.*

8. *Paraffin Oil.*

9. *Nessler's Solution* (Folin and Wu's Formula).—A stock solution of mercuric potassium iodide is prepared by dissolving 150 gm. of potassium iodide in 100 cc. of warm water, adding 200 gm. of mercuric iodide, stirring until the latter is dissolved, and diluting to a volume of about 1 liter. This is allowed to settle when it is filtered and diluted to a final volume of 2 liters. It is advisable to make this in large volumes since a second sediment may form which requires a long time to settle.

From this stock solution the final Nessler's solution is made as follows: A saturated solution of caustic soda is prepared and the clear supernatant fluid is decanted. This is diluted to a concentration of about 10 per cent. While the saturated solution contains about 55 gm. of the alkali per 100 cc., it is wise to check the concentration by titration against a standard acid. Into a large bottle is introduced 3500 cc. of this 10 per cent. sodium hydrate solution with 750 of the mercuric potassium iodide solution and 750 cc. of water, making the total volume of solution 5000 cc.[1]

Procedure.—Blood is obtained and oxalated as for other chemical procedures (see page 167). Protein is precipitated by sodium tungstate and sulphuric acid as described previously (see page 173). Five cc. of the filtrate (corresponding to 0.5 cc. of blood) are transferred to a clean dry Pyrex ignition tube with a capacity of about 75 cc. The tubes should be kept for this purpose alone and should be free from mercury or precipitated Nessler's solution. To the blood filtrate are added 2 drops of the pyrophosphate solution and 0.5 to 1 cc. of urease solution, when the tube is immersed for five minutes in a beaker of warm water, the temperature of which does not exceed 55° C.

The ammonia may be distilled into 2 cc. of twentieth-normal hydrochloric acid contained in a second test-tube, or it may be liberated and drawn by air current into a second tube containing 2 cc. of twentieth-normal hydrochloric acid. The method for arranging aspirating outfit for the latter method has been given in describing the preceding technic for determining urea (see page 179). If aspiration be employed, add 1 or 2 cc. of 10 per cent. sodium hydrate to the hydrolyzed blood mixture just before aspirating. The calculation is the same in either case.

Distillation may be performed with the aid of very simple apparatus. The tubes containing the treated specimen which is to be distilled and that containing the acid are both fitted with rubber stoppers, each pierced by a single hole. The stopper for the second or receiving test-tube should be cut with a fairly deep notch to permit the escape of steam and air. A piece of glass tubing is bent into a V form, so that the arms will be unequal. The shorter one is inserted through the stopper of the tube containing the specimen, the longer one through the stopper in the receiving tube. The end of the first arm should come just below the stopper while the tip of the second arm should dip into the acid in the receiving tube. No condensing apparatus is needed.

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[1] On account of the difficulty of obtaining high-grade mercuric iodide, Folin and Wu offer the following method of preparing the double iodide solution: Place 150 gm. of potassium iodide and 110 gm. of iodine in a 500 cc. Florence flask. Add 100 cc. of water and an excess of metallic mercury (140 to 150 gm.). Shake the flask continuously for ten to fifteen minutes until the dissolved iodine has nearly disappeared. When the red iodine solution has begun to pale visibly but while it is still red, cool the hot solution in running water while shaking until the red color of the iodine has been replaced by the greenish color of the double iodide. This usually requires about fifteen minutes. Decant the clear solution from the surplus mercury. Wash the mercury liberally with water, adding the washings to the solution. Dilute mixture of solution and washings to a volume of 2 liters. This preparation may be diluted with alkali exactly as the other solution of mercuric potassium iodide, but is usually clear enough for immediate dilution. The cost is less, and impurities are avoided.

To the hydrolyzed blood filtrate is added a dry pebble, 2 cc. of saturated borax solution, and a drop or two of paraffin oil. The rubber stoppers are inserted firmly in both test-tubes, and the hydrolyzed filtrate is boiled moderately fast over a microburner for four minutes. The size of the flame should not be cut down during this time nor the boiling should be so brisk that emission of steam from the receiving tube begins before the end of three minutes. At the end of four minutes the stopper is removed from the receiving tube and the end of the V-shaped delivery tube is held near the top of the receiving test-tube, while distillation is continued for one minute. The lower part of the delivery tube is rinsed off with a little water into the receiving tube.

The distillate should be cooled with running water, when it is diluted to about 20 cc. Then 2.5 cc. of Nessler's solution are added and the volume is brought up to exactly 25 cc. The unknown is placed in the colorimeter and compared with a standard nitrogen solution. This is prepared by placing 0.3 mg. of nitrogen in a 100 cc. volumetric flask and adding 10 cc. of Nessler's solution and then diluted to 100 cc.

Résumé of Method.—(Steps one to three inclusive are the same as those used for securing protein-free filtrate for blood sugar determinations, see page 173. Amount of blood used depends upon number of tests to be done.)

1. Place one volume of oxalated blood in an Erlenmyer flask.
2. Add (*a*) 7 volumes of distilled water.
   (*b*) 1 volume of 10 per cent. sodium tungstate.
   (*c*) 1 volume of two-thirds normal sulphuric acid.
   Rotate flask constantly while making additions.
3. Filter when coagulation is complete.
4. Place 5 cc. of protein-free filtrate in Pyrex ignition tube.
5. Add (*a*) 2 drops of pyrophosphate solution.
   (*b*) 0.5 to 1 cc. of urease solution.
6. Immerse tube in warm water for five minutes.
7. Arrange distilling apparatus, placing 2 cc. $\frac{N}{20}$ HCl in receiving tube.
8. Add to blood (*a*) a dry pebble.
   (*b*) 2 cc. of saturated borax solution.
   (*c*) a drop of paraffin oil.
9. Insert stoppers in both tubes of distilling apparatus, and boil blood mixture over microburner for five minutes as directed.
10. Rinse tip of delivery tube into receiving tube with a little distilled water.
11. Cool distillate and add about 20 cc. of water.
12. To distillate, add
    (*a*) 2.5 cc. of Nessler's solution (Folin and Wu's formula).
    (*b*) Distilled water to make volume of 25 cc.
13. Prepare standard as follows:
    (*a*) 5 cc. of standard nitrogen solution.

(*b*) 10 cc. of Nessler's solution.
(*c*) Distilled water to make volume of 100 cc.

14. Compare in colorimeter.

Calculation.—Multiply 20 (the height of the standard) by 15 and divide by the reading of the colorimeter tube containing the unknown to obtain the amount of urea nitrogen in terms of mg. per 100 cc. of blood.

Findings.—The normal findings are from 12 to 15 mg. urea nitrogen per 100 cc. of blood. An increase in the quantity (nitrogen retention) is seen in cases of nephritis, the amount of increase varying to some extent with the gravity of the process.

NON-PROTEIN NITROGEN.

Myers's Method (Modified from those of Folin and Denis and Greenwald).—*Principle.*—The proteins are removed by precipitation with trichloracetic acid, and the filtrate is boiled in the presence of an oxidizing agent with concentrated sulphuric acid, so that the nitrogenous bodies are converted to ammonium sulphate. This is decomposed by the action of an alkali with the formation of ammonia, which is liberated by aëration and aspirated into a dilute solution of sulphuric acid, where ammonium sulphate is formed. The amount of this is determined after Nesslerization by colorimetry.

Reagents Required.—All of those required for the determination of urea will be needed with the exception of urease, and in addition potassium sulphate, concentrated sulphuric acid, 10 per cent. aqueous solution of copper sulphate, trichloracetic acid (2.5 per cent. dilution in water).

Procedure.—In a flask are placed 20 cc. of 2.5 per cent. trichloracetic acid and to it are added slowly 5 cc. of oxalated blood, stirring the mixture with a glass rod during the process of addition. The mixture is allowed to stand for thirty minutes, when it is filtered. Into the ignition tube is placed 10 cc. of the filtrate (equivalent to 2 cc. of blood). To the filtrate are added 1 cc. concentrated sulphuric acid, 1 gm. potassium sulphate, and 2 drops 10 per cent. solution of copper sulphate. The mixture is then boiled over a small micro flame. Provision should be made for the removal of fumes as shown in the illustration (Fig. 56). Heating should be allowed to proceed until digestion is complete. It is necessary to evaporate practically all of the fluid. The small residue chars to a brown color. This must be heated still further until the solution is practically colorless or is a faint bluish green. The tube is allowed to cool for a moment or so, but not long enough to permit solidification of the solution, when about 3 cc. of distilled water are added.

The solution is now to be treated with a strong alkali and aërated to aspirate the ammonia, as in the determination of urea. In the tube are placed 5 cc. of a saturated solution of sodium carbonate and 1 cc. of amyl alcohol and the rubber stopper is inserted tightly. A cylindrical 100 cc. graduate should have been charged previously with 2 cc. of decinormal sulphuric acid and 15 cc. of distilled water. The ammonia liberated from the test-tube is aspirated by the air current into the dilute sulphuric acid. Aëration should be allowed to proceed for thirty to forty-five minutes. At the end of this time Nesslerization, the preparation of the standard solution, the reading of the result, and the calculation may be carried out as described for the determination of urea in the blood (q. v.).

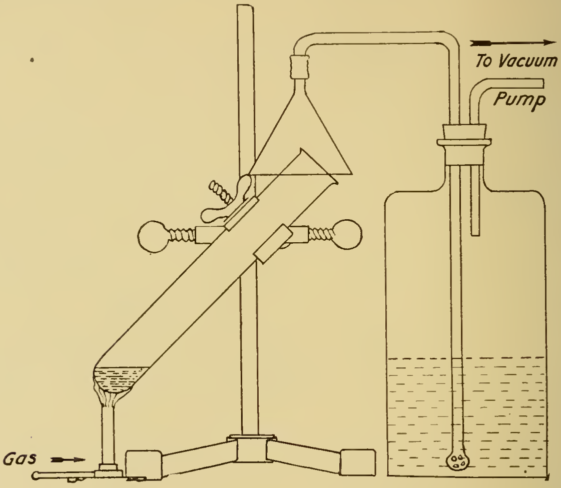

Fig. 56.—Arrangement for apparatus for determination of non-protein nitrogen of blood with arrangement for removing fumes. (After Myers.)

Résumé of Method.—

1. Place 20 cc. of 2.5 per cent. trichloracetic acid in a small flask.
2. Add slowly 5 cc. of oxalated blood, stirring with glass rod after each addition.
3. Let stand thirty minutes, then filter.
4. Place 10 cc. of filtrate in ignition tube.
5. Add (*a*) 1 cc. concentrated sulphuric acid.
    (*b*) 1 gm. potassium sulphate.
    (*c*) 2 drops of 10 per cent. copper sulphate.
6. Boil tube over small micro flame until brown color changes to white or faint bluish-green.

7. Cool for a moment or two, avoiding solidification.
8. Add 3 cc. distilled water.
9. Place tube inside cylinder *A* of the aërating outfit.
10. In receiving cylinder *B*, place 2 cc. of $\frac{N}{10}$ H_2SO_4 and 15 cc. of distilled water.
11. Add to tube in cylinder *A*, 5 cc. of saturated solution of sodium carbonate.
12. Stopper cylinders *A* and *B* connect up the apparatus, and start the air current.
13. Allow aëration to proceed for thirty to forty-five minutes.
14. Take cylindrical graduate *C* of the same size as *B* and mix in it the standard ammonium sulphate solution.

Treat the contents of cylinders *B* and *C* as follows:

	CYLINDER B (CONTAINS THE UNKNOWN).	CYLINDER C (CONTAINS THE STANDARD).
15.	Contains substances placed in it in step 10 plus the liberated ammonia.	Place in cylinder 5 cc. standard solution, ammonium sulphate (contains 1 mg. nitrogen).
16.		Add 20 cc. distilled water.
17.	Add 20 per cent. dilution of Nessler's solution to maximum color.	Add 20 cc. of 20 per cent. dilution of Nessler's solution.
18.	Dilute to 30, 50, or 100 cc. to match roughly the color of *C*.	Bring total volume to 100 cc. with distilled water.

19. Compare contents of two cylinders in colorimeter.

Folin and Wu's Method.—*Principle.*—Protein-free filtrate is treated with an acid digestion mixture by means of which the nitrogen bodies are broken down. The resulting solution is directly Nesslerized and compared with a standard nitrogen solution, similarly Nesslerized.

Reagents Required.—1. *Sodium Tungstate.*—See page 173.

2. *Two-thirds Normal Sulphuric Acid.*

3. *Sulphuric-phosphoric Acid Digestion Mixture.*—300 cc. of phosphoric acid syrup (about 85 per cent. H_3PO_4) are mixed with 100 cc. of concentrated sulphuric acid in a tall cylinder. This is covered to prevent the absorption of ammonia and set aside to permit sedimentation of calcium sulphate. Sedimentation is slow, but in a week or so the top part is clear enough to permit the removal of 50 or 100 cc. by means of a pipette. To 100 cc. of the clear acid mixture is added to 10 cc. of 6 per cent. copper sulphate solution and 100 cc. of water.

4. *Standard Nitrogen Solution.*—5 cc. containing 3 mg. of nitrogen. See page 180.

5. *Nessler's Solution.*—Folin and Wu's formula. See page 180.

Procedure.—Blood is obtained and oxalated as for other chemical procedures (see page 167). Protein is precipitated by sodium tungstate and sulphuric acid as described previously (see page 173). Five cc. of the filtrate (corresponding to 0.5 cc. of blood) are transferred to a dry clean Pyrex ignition tube having a capacity of about 75 cc. (200 × 25 mm.). The tube should be graduated at 35 and 50 cc. by marks on two sides. Folin emphasizes the importance of having the tube thoroughly dry to prevent bumping. One cc. of the sulphuric-phosphoric acid mixture is added, a dry quartz pebble placed in the tube, and the mixture boiled over a microburner until the characteristic dense acid fumes fill the tube. These are usually seen in from three to seven minutes. When definitely present, the size of the flame is reduced so that the contents are just boiling and the mouth of the flask closed with a small watch glass or tiny Erlenmyer flask, after which the boiling is continued for two additional minutes. When solution is almost colorless, it should be boiled until practically all trace of color has disappeared. The contents are cooled for seventy to ninety seconds and 15 to 25 cc. of water are added. After cooling further to about room temperature, water is added to exactly the 35 cc. mark.

The standard should be prepared now, placing 5 cc. of the nitrogen standard in 100 cc. volumetric flask, adding in turn 2 cc. of the sulphuric-phosphoric acid mixture, 50 cc. of water, and 30 cc. of Nessler's solution (Folin and Wu's formula). The flask is filled with water to the 100 cc. mark and the contents are mixed.

15 cc. of Nessler's solution are added to the unknown in the tube. Should turbidity develop, a portion is centrifuged and the supernatant clear fluid compared in the colorimeter with the unknown.

Résumé of Method.—(Steps 1 to 3 inclusive are the same as those used for securing *protein-free filtrate for blood-sugar determinations.*) Amount of blood treated depends upon number of tests to be done.

1. Place 1 volume of well-mixed, oxalated blood in an Erlenmyer flask.
2. Add (*a*) 7 volumes of distilled water.
   (*b*) 1 volume of 10 per cent. sodium tungstate.
   (*c*) 1 volume of two-thirds normal H_2SO_4.
   Rotate flask constantly while making additions.
3. Filter when coagulation is complete.
4. Transfer 5 cc. of filtrate to dry 75 cc. graduated ignition tube.
5. Add a dry quartz pebble; boil vigorously over microburner until dense acid fumes fill test-tube.
7. Reduce flame so as to keep at boiling point only; close test-tube mouth with watch-glass.
8. Continue heating two minutes. (Solution must be nearly colorless.)
9. Cool seventy to ninety seconds; add 15 to 25 cc. of water.
10. Cool to about room temperature and add water to 35 cc. mark.
    Prepare standard and unknown as follows:

	TUBE WITH UNKNOWN.	FLASK WITH STANDARD.
11.		5 cc. of standard nitrogen solution (= 0.3 mg. N.).
12.		Add 2 cc. of sulphuric-phosphoric acid mixture.
13.		Add 50 cc. of water.
14.	Add 15 cc. of Nessler's solution.	Add 30 cc. of Nessler's solution.
15.	If turbid, centrifuge a portion and decant clear fluid for comparison.	Bring to 100 cc. mark with distilled water and mix.
16.	Compare in colorimeter.	

Calculation.—The reading of the standard is divided by the reading of the unknown and multiplied by 30 to give the grams of non-protein nitrogen per 100 cc. of blood.

Findings.—Normally 25 to 30 mg. of non-protein nitrogen are present in 100 cc. of blood. The amount may be increased in the same conditions which lead to retention of urea in the blood (q. v.). In fact, many workers prefer not to estimate the non-protein nitrogen but rather to estimate the urea in the blood, since the latter comprises an almost constant proportion of the former and the procedure for its determination is much simpler.

BLOOD CREATININE.

Myers and Lough's Method (Modified from Folin).—*Principle.*—The proteins are precipitated from the laked blood by saturation with picric acid. They are removed by centrifugalization and filtering, and to the filtrate is added a dilute alkali. This results in the formation of sodium picrate, which is reduced by the creatinine. The amount of creatinine is determined by the amount of the color change, compared with the effects of the same amount of alkali on picric acid solutions of creatinine of known strength.

Reagents Required.—1. Standard solutions of creatinine three in number, made up to contain respectively 0.3 mg., 0.5 mg. and 1.0 mg. to 100 cc. of a saturated solution of picric acid.

2. Ten per cent. aqueous solution of sodium hydrate.

3. Picric acid, pure, crystals.

For method of testing purity of picric acid and for purifying it, see page 442. For method of preparing creatinine standards, see page 444.

Procedure.—Blood is obtained and the proteins are precipitated by saturation by the addition of picric acid crystals, as directed in the method for the determination of sugar (see page 169). The proteins are separated by centrifugalization and filtration in the same way. If both sugar and creatinine determinations are to be made, portions of the same filtrate may be used for the two tests. Into a 10 cc. graduated cylinder (Fig. 54, *i*) are placed 10 cc. of the filtrate and into each of the other three cylinders are placed 10 cc. of different standard solutions, containing respectively, 0.3 mg., 0.5 mg., and 1.0 mg of creatinine to 100 cc. of saturated picric acid solution. To each cylinder is then added 0.5 cc. of a 10 per cent. aqueous solution of sodium hydrate. The contents of the cylinders are mixed by placing the thumb over the ends and inverting gently, when they are allowed to stand for exactly eight minutes, at the end of which time the reading should be made promptly. That standard should be selected whose shade most closely approximates that of the unknown. The standard is then placed in the left hand cup of the colorimeter and the unknown in the right hand cup. With the cup containing the standard set at 10 mm., the reading is taken of the unknown.

Résumé of Method.—

1. To 5 cc. of blood in heavy-walled 50 cc. centrifuge tubes add 20 cc. of distilled water.
2. Add dry picric acid to saturation stirring thoroughly with glass rod.
3. Let stand five minutes, then centrifugalize.
4. Filter.
5. Place 10 cc. filtrate in a 10 cc. graduate cylinder. Use three other graduated cylinders of same size for three standard solutions.

CYLINDER A	CYLINDER B.	CYLINDER C.	CYLINDER D.
(Contains the unknown).	(0.3 mg. creatinine per 100 cc.).	(0.5 mg. creatinine per 100 cc.).	(1.0 mg. creatinine per 100 cc.).
6. Place 10 cc. of filtrate.	Place 10 cc. of proper standard solution.	Place 10 cc. of proper standard solution.	Place 10 cc. of proper standard solution.
7. Add 0.5 cc. 10 per cent NaOH solution.	Add 0.5 cc. 10 per cent. NaOH solution.	Add 0.5 cc. 10 per cent. NaOH solution.	Add 0.5 cc. 10 per cent. NaOH solution.

8. Put thumb over end of each cylinder in turn, invert to mix, and let all stand exactly eight minutes. Then select standard whose color most nearly matches that of unknown. Compare this with the unknown (cylinder *A*) in the colorimeter.

Calculation.—The result may be determined by the formula

$$X = 5\,\frac{(SA)}{R}$$

when S represents the reading of the standard; R, the reading of the unknown; A, mg. creatinine in 100 cc. of the standard; and X, mg. creatinine in 100 cc. of blood.

Folin and Wu's Method.—*Principle.*—A protein-free filtrate is obtained after coagulation with tungstic acid. This is treated with a solution of picric acid in the presence of an alkali and the color change produced is compared with that caused by a standard solution of creatinine on a similar alkaline picrate solution.

Reagents Required.—1. *Sodium Tungstate* (10 per cent. solution).

2. *Two-thirds Normal Sulphuric Acid.*

3. *Saturated Solution of Picric Acid.*—See page 442.

4. *Sodium Hydrate* (10 per cent. solution).

5. *Standard Creatinine Solution.*—See "Preparation of Creatinine Zinc Chloride," page 444, for the preparation of the basic solution. This is made by dissolving 1.6106 gm. creatinine zinc chloride in sufficient decinormal hydrochloric acid to make 1 liter; 1 cc. of this solution contains 1 mg. of creatinine; 6 cc. are placed in a liter flask with 10 cc. of normal hydrochloric acid. Water is added to bring the volume to 1000 cc. The contents of the flask are mixed and transferred to a bottle for storage, adding 4 or 5 drops of xylene or toluene. 5 cc. of this solution contains 0.03 mg. of creatinine and when diluted with 15 cc. of water as directed below, furnish a standard suitable for most blood specimens. When there is marked retention, 10 cc. of standard may be diluted with 10 cc. of water, the standard now containing 0.06 mg.; or 15 cc. with 5 cc. of water, the standard containing 0.09 mg.

Procedure.—Blood is obtained and oxalated as for other chemical procedures (see page 167). Protein is precipitated by sodium tungstate and sulphuric acid as described previously (see page 173); 25 cc. of a saturated solution of picric acid (see page 442) are placed in a small clean flask and mixed with 5 cc. of 10 per cent. sodium hydrate. Then two small flasks are taken. In one are placed 10 cc. of the protein-free blood filtrate (corresponding to 1 cc. of blood), while in the other are placed 5 cc. of the standard creatinine solution, containing 0.03 mg. creatinine and 15 cc. of water. Next 5 cc. of freshly prepared alkaline picrate are added to the creatinine solution. After standing eight to ten minutes, the contents of the two flasks are compared in the colorimeter.

Résumé of Method.—(Steps one to three inclusive are the same as those used for securing protein-free filtrate for blood-sugar determinations, see page 173. (Amount of blood treated depends upon number of tests to be done.)

1. Place 1 volume of oxalated blood in an Erlenmyer flask.
2. Add (*a*) 7 volumes of distilled water.
    (*b*) 1 volume of 10 per cent. sodium tungstate.
    (*c*) 8 volumes of two-thirds normal sulphuric acid.
   Rotate flask constantly while making additions.
3. Filter when coagulation is complete.
4. Employ a portion of filtrate as shown below (Step 6).
5. Make fresh alkaline picrate solution by mixing 25 cc. of saturated solution picric acid with 5 cc. sodium hydrate, 10 per cent. Use in Step 8 as shown below.

	Flask A (Contains Unknown).	Flask B (Contains Standard).
6.	Place in flask 10 cc. of blood blood filtrate from Step 3.	Place in flask 5 cc. of standard creatinine solution (0.3 mg.).
7.		Add 15 cc. of water.
8.	Add 5 cc. of alkaline picrate solution from Step 5.	Add 10 cc. alkaline picrate solution from Step 5.
9.	Let stand eight to ten minutes.	
10.	Compare in colorimeter.	

Calculation.—Multiply the reading of the standard by 1.5 and divide by the reading of the unknown to obtain the number of milligrams of creatinine per 100 cc. of blood. If the double strength were employed, this result should be multiplied by two.

Findings.—The normal findings are from 1 to 2 mg. creatinine in 100 cc. of blood. Myers and Lough concluded that the creatinine very seldom rose above 2.5 mg. per 100 cc. of blood unless there were renal involvement. Creatinine values of from 3.0 to 5 mg. should be regarded as decidedly unfavorable, and figures over 5.0 mg. probably indicate an early fatal termination.

CREATININE PLUS CREATININE.

Folin and Wu's Method.—*Reagents Required.*—The same as for the determination of creatinine by the Folin and Wu method and, in addition, normal hydrochloric acid.

Procedure.—Five cc. of the protein-free blood filtrate obtained after coagulation with tungstic acid (see page 173) are placed in a test-tube graduated at 25 cc. 1 cc. of normal hydrochloric acid is added and the tube heated in an autoclave, either at 130° C. for twenty minutes, or at 155° C. for ten minutes after covering the mouth with tin-foil. After cooling 5 cc. of alkaline picrate solution are added. At the same time the standard should be prepared by placing 20 cc. of creatinine standard solution in a 50 cc. volumetric flask, mixing with it 2 cc. of normal hydrochloric acid and 10 cc. of alkaline picrate solution. Both standard and unknown are allowed to stand for eight to ten minutes, when water is added to bring the volume of the unknown to 25 cc. and that of the standard to 50 cc. Comparison is made immediately in the colorimeter.

Calculation.—The reading of the standard, multiplied by 6 and divided by the reading of the unknown, gives the amount of total creatinine in mg. per 100 cc. of blood.

Normal Findings.—The authors state that the normal value for "total creatinine" as determined by this method is about 6 mg. per 100 cc. of blood.

URIC ACID.

Folin and Wu's Method.—*Principle.*—The proteins are removed from the blood by filtration after the precipitation with tungstic acid and the purins in the filtrate are then precipitated with silver lactate. The uric acid is set free from the precipitate by dissolving it in acidulated 10 per cent. sodium chloride solution. On the addition of phosphotungstic acid solution (Folin-Denis uric acid reagent), uric acid gives a delicate color reaction, which is used as a quantitative test. For the purpose of comparison a standard solution containing a known amount of uric acid is similarly treated.

Reagents Required.—1. *Folin-Denis Uric Acid Reagent* (Benedict's modification):

Sodium tungstate	100 gm.
Hydrochloric acid (concentrated)	20 cc.
Phosphoric acid (85 per cent.)	30 "
Distilled water q. s.	1000 "

The sodium tungstate, hydrochloric acid, and phosphoric acid are boiled with 750 cc. of water for two hours, preferably under a reflux condenser. After cooling, the volume is made up to a liter with distilled water.

2. *Folin's Silver Lactate Solution.*—This is a 5 per cent. solution of silver lactate in 5 per cent. solution lactic acid.

3. *Potassium Cyanide.*—A 5 per cent. solution in water. Folin states that sodium cyanide may be used instead.

4. *Sodium Tungstate* (10 per cent. solution).—See page 173.

5. *Sulphuric Acid Solution, Two-thirds Normal.*

6. *Standard Uric Acid Sulphite Solution.*—Make 1 to 3 liters of 20 per cent. solution of sodium sulphite, allow to stand overnight, and filter. Dissolve 1 gm. uric acid in about 125 cc. of 0.4 per cent. lithium carbonate solution and dilute to a volume of 500 cc. Transfer portions of 50 cc. each to a series of volumetric flasks of 1 liter capacity. This amount contains 100 mg. of uric acid. To each flask add 200 to 300 cc. of water, then 500 cc. of filtered sodium sulphite solution, and finally make up to volume with water. Mix well. Fill with the solution a series of 200 cc. bottles, stoppering tightly with rubber stoppers. Folin has found that the solution in a bottle which is opened daily will keep at least three to four months.

7. *Sodium Sulphite* (10 per cent. solution).—The surplus 20 per cent. sodium sulphite solution left after preparing the above uric acid standard is diluted with an equal amount of water to give a concentration of 10 per cent. and is transferred to a series of small, tightly stoppered bottles. This is added to the unknown to offset the sulphite content of the standard.

8. *Sodium Carbonate* (20 per cent. solution).

9. *Sodium Chloride Solution* (10 per cent. solution).—This is prepared by dissolving the salt in decinormal hydrochloric acid.

Procedure.—The proteins in a specimen of blood are precipitated with tungstic acid according to the method described elsewhere (see page 173).

Twenty cc. of the filtrate are now transferred to a large 50 cc. centrifuge tube. If a centrifuge (Fig. 57) of this capacity be not available, two small 15 cc. tubes may be used, placing 10 cc. of the filtrate in each tube; or the amount may be run down in two lots of 10 cc. each. 4 cc. of 5 per cent. silver lactate solution in 5 per cent. lactic acid are added to 20 cc. of filtrate, or 2 cc. to each 10 cc. amount. The mixture is stirred and centrifugalized. A drop of silver lactate solution is added to the supernatant fluid, which should be practically clear and should show no turbidity on the addition of the last drop of the silver solution.

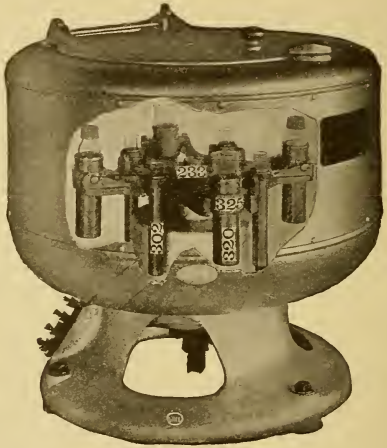

Fig. 57.—Type of centrifuge suitable for serological work.

The supernatant fluid is removed by decantation as thoroughly as possible and discarded. To the precipitate from 20 cc. are added 2 cc. of 10 per cent. solution of sodium chloride in $\frac{N}{10}$ HCl. If the two portions of 10 cc. be used, then 1 cc. should be added to each portion. This is stirred well with a glass rod, about 5 or 6 cc. of water are added, again stirred, and centrifugalized. The supernatant fluid, containing the dissolved uric acid, is poured into an accurately graduated 25 cc. volumetric flask. If the precipitate were run down in two lots of 10 cc. each, the fluids should be mixed. Now should be added in order 1 cc. of 10 per cent. sodium sulphite solution, 0.5 cc. of 5 per cent. sodium cyanide solution, and 3 cc. of 20 per cent. sodium carbonate solution.

Simultaneously two standard uric acid solutions should be prepared as follows: Transfer to two 50 cc. volumetric flasks 1 cc. and 2 cc. respectively of the standard uric acid sulphite solution. Add 1 cc. of 10 per cent. sodium sulphite to the first flask. Then add to each flask 4 cc. of acidified salt solution, 1 cc. of 5 per cent. sodium cyanide solution, and 6 cc. of sodium carbonate solution, diluting with water to about 45 cc.

When the two standards and the unknown have been prepared in this way, they are ready for the addition of Folin and Denis's uric acid reagent, of which 0.5 cc. is added to the unknown and 1 cc. to the standards. The contents are mixed and after standing for ten minutes, all flasks are filled to the marks with water and again mixed, when the unknown is compared in the colorimeter with the standard which matches more nearly in color.

Résumé of Method.—(Steps 1 to 3 inclusive are the same as those used for securing protein-free filtrate for blood-sugar determinations see page 173.)

1. Place 1 volume of well-mixed, oxalated blood in an Erlenmyer flask.
2. Add (*a*) 7 volumes of distilled water.
   (*b*) 1 volume of 10 per cent. sodium tungstate.
   (*c*) 1 volume of two-thirds normal H_2SO_4.
   Rotate flask constantly while making additions.
3. Filter when coagulation is complete.
4. Transfer 20 cc. of filtrate to 50 cc. centrifuge tube.
5. Add 4 cc. of silver lactate solution and stir well.
6. Add 5 cc. of water, stir again, and centrifugalize.
7. Add another drop of silver lactate. Supernatant fluid should be clear. If cloudy, centrifugalize again.
8. Decant and discard supernatant fluid.
9. Add 2 cc. of acidified salt solution to sediment, stir and centrifugalize.
10. Transfer to 25 cc. volumetric flask (flask A). Treat as shown below.
11. Take two 50 cc. volumetric flasks and prepare as shown below.

	Flask A	Flask B	Flask C
	(Unknown).	(First Standard).	(Second Standard).
12.	Dissolved precipitate as shown in Step 10.	Put in 1 cc. of uric acid sulphite standard sol.	Put in 2 cc. of uric acid sulphite standard sol.
13.	Add 1 cc. of 10 per cent. sodium sulphite.	Add 1 cc. of 10 per cent. sodium sulphite.	
14.		Add 4 cc. of acidified salt sol.	Add 4 cc. of acidified salt sol.
15.	Add 0.5 cc. of 5 per cent. sodium cyanide.	Add 1 cc. of 5 per cent. sodium cyanide.	Add 1 cc. of 5 per cent. sodium cyanide.

	Flask A	Flask B	Flask C
16.	Add 3 cc. of 20 per cent. sodium carbonate.	Add 6 cc. of 20 per cent. sodium carbonate.	Add 6 cc. of 20 per cent. sodium carbonate.
17.		Dilute with water to about 45 cc.	Dilute with water to about 45 cc.
18.	Add 0.5 cc. of Folin-Denis reagent and mix.	Add 1 cc. of Folin-Denis reagent and mix.	Add 1 cc. of Folin-Denis reagent and mix.
19.	Bring volume to 25 cc. with water and mix.	Bring volume to 50 cc. with water and mix.	Bring volume to 50 cc. with water and mix.

20. Let all flasks stand ten minutes and compare in colorimeter the contents of A with the standard solution which matches more closely.

Calculation.—The concentration of the unknown may be computed with this formula, when the weaker standard is used:

$$\text{Mg. of uric acid per 100 cc. of blood} = \frac{2.5\ S}{R}$$

S represents the reading of the standard and R that of the unknown. When the stronger standard is used, the result obtained by the above formula is multiplied by 2.

Myers's Modification of Folin's Method.—*Principle.*—The principle is essentially the same as in Folin's method except that a different standard is employed.

Reagents Required.—The same as for Folin's method except that sodium sulphite is not needed, and that Benedict's standard is employed.

Benedict's Standard Uric Acid Solution.

Nine grams of pure crystalline hydrogen di-sodium phosphate and 1 gram of dihydrogen sodium phosphate are dissolved in 300 cc. of hot distilled water. This solution should be filtered and the volume made up to 500 cc. with distilled water. The clear, warm solution is poured onto 200 mg. of uric acid, suspended in a little water in a volumetric flask of one liter capacity. The flask should be stoppered and the mixture shaken until the uric acid is completely dissolved. Then 1.4 cc. of glacial acetic acid is added by accurate measurement and the total volume is brought to one liter with distilled water. About 5 cc. of chloroform should be added as a preservative. This solution should be prepared freshly about every two months. One cc. of this solution contains 0.2 mg. of uric acid.

Procedure.—The procedure is the same as for the preceding method up to and including the step when the precipitate obtained with silver lactate is treated with acidified salt solution and centrifugalized. After centrifugalization, the supernatant clear fluid is decanted into an accurately graduated 25 cc. cylinder, and the standard solution is prepared.

With an accurate pipette, Ostwald or Folin type, 1 cc. of the uric acid standard is placed in another accurately graduated 25 cc. cylinder. Four cc. of 10 per cent. sodium chloride made up on $\frac{N}{10}$ HCl are added with enough water to make the volume essentially the same as that of the unknown and a drop of 5 per cent. potassium or sodium cyanide. To the standard there should be added 1 cc. of the Folin-Denis uric acid reagent and 6 cc. of saturated solution of sodium carbonate, while to the unknown there should be added 0.5 cc. uric acid reagent and 3 cc. saturated solution of sodium carbonate. The color is allowed to develop for five minutes, when the standard is diluted to 25 cc. with distilled water. The unknown is likewise diluted with distilled water until its color is of similar intensity. The color of the unknown is compared with that of the standard by the colorimeter, in the manner described for blood sugar, creatinine, etc.

Résumé of Method.—(Steps one to three inclusive are the same as those used for securing *protein-free filtrate for blood sugar determinations.*)

1. Place 5 cc. of well-mixed, oxalated blood in Erlenmeyer flask.
2. Add (*a*) 35 cc. of distilled water.
   (*b*) 5 cc. of 10 per cent. sodium tungstate.
   (*c*) 5 cc. of two-thirds normal H_2SO_4.
   Rotate flask constantly while making additions.
3. Filter when coagulation is complete.
4. Transfer 20 cc. of filtrate to large 50 cc. centrifuge tube.
5. Add 4 cc. of 5 per cent. silver lactate solution (made up in 5 per cent. lactic acid). Stir well.
6. Add 5 or 6 cc. of water; stir again.
7. Centrifugalize.
8. Pour off and discard supernatant fluid.
9. To the precipitate add 2 cc. of 10 per cent. sodium chloride (made up in $\frac{N}{10}$ HCl).
10. Stir with glass rod, add 5 or 6 cc. water, and stir again.
11. Centrifugalize.
12. Pour supernatant fluid into graduated 25 cc. cylinder (*A*). Treat this as outlined below. Sediment is discarded.
13. Take a similar 25 cc. graduated cylinder and in it prepare standard as outlined below.

	CYLINDER A (UNKNOWN).	CYLINDER B (STANDARD).
14.	Contents as directed in step 13.	Put in 1 cc. of standard uric acid solution.
15.		Add 4 cc. of 10 per cent. NaCl (made up in $\frac{N}{10}$ HCl).
16.		Add distilled H_2O to make volume same as in cylinder *A*.

CYLINDER A (UNKNOWN).	CYLINDER B (STANDARD).
17. Add a drop of 5 per cent. NaCN solution.	Add a drop of 5 per cent. NaCN solution
18. Add 0.5 cc. of Folin-Denis uric acid reagent.	Add 1 cc. of Folin-Denis uric acid reagent.
19. Add 3 cc. of saturated solution Na_2CO_3.	Add 6 cc. of saturated solution Na_2CO_3.
20. Allow to stand five minutes.	Allow to stand five minutes.
21. Add water to approximately match intensity of color in standard which has been diluted to 25 cc.	Add water to bring volume to 25 cc.

22. Compare contents of two cylinders in colorimeter.

Calculation.—The concentration of the unknown solution may be computed with the aid of the formula:

$$X = \frac{4\,SA}{5\,RB}$$

X is the number of mg. uric acid per 100 cc. of blood, while S represents the depth of colorimeter cup containing the standard, R the reading of the unknown, A the number of cc. to which the unknown was diluted for color comparison and B the number of cc. of blood represented by the amount of filtrate employed.

Findings.—The normal findings run from 2 to 3 mg. per 100 cc. of blood. The amount is increased in gout. The determination of uric acid has been advocated as a valuable guide in early nephritis, since an increase is seen in the blood before the other non-protein nitrogen elements show elevation over normal figures. Amounts as high as 12 mg. per 100 cc. of blood in early interstitial nephritis have been reported.

GENERAL CONSIDERATIONS REGARDING THE RETENTION OF URIC ACID, UREA AND CREATININE.

In *gout* the blood shows a high uric content. This is seen also in cases of early nephritis, though in such instances there is usually an increase in the amount of urea also. Myers and Chace have studied the retention of the various nitrogenous factors in cases of *nephritis* and describe what they term the "staircase retention." According to their investigations, uric acid increase is seen in the less severe cases. In the next group, the blood urea is increased as well as the uric acid, while in the most severe type, marked by fatal termination, there is an increase in the creatinine as well as in the urea and uric acid. Myers states that the creatinine is more easily eliminated than uric acid or urea and that there is usually not an appreciable increase in the blood until the blood urea has doubled, or more than doubled, the normal amount.

STAIRCASE RETENTION OF URIC ACID, UREA, AND CREATININE IN NEPHRITIS.

Date, 1915–16.	Case.	Age.	Sex.	Diagnosis.	Condition.	Mg. per 100 cc. of blood.			Phthalein, two hours, per cent.	Systolic blood pressure.	Urine.	
						Uric acid.	Urea N.	Creatinine.			Albumin.	Casts.
I												
Sept. 17	H. L.	23	♂	Pulmonary tuberculosis	Unchanged	6.5	16	2.7	58	130	++	+
Aug. 10	E. H.	41	♂	Pericarditis	Unchanged	5.6	13	2.1	45	150	−	−
Oct. 12	F. D.	45	♂	Interstitial nephritis	Unchanged	5.5	12	2.5	37	185	−	+
Mar. 6	B. D.	35	♀	Diffuse nephritis	Unchanged	9.6	19	2.4	45	175	+	+
II												
Aug. 11	J. J.	65	♂	Early interstitial nephritis	Unchanged	9.5	25	2.5	13	185	+	+
July 21	D. S.	56	♂	Early interstitial nephritis	Unchanged	6.6	24	3.3	26	185	−	+
Sept. 21	D. D.	52	♂	Early interstitial nephritis	Unchanged	8.7	20	3.6	20	100	+	+
Aug. 3	C. M.	54	♂	Early interstitial nephritis	Unchanged	6.3	31	2.0	23	150	−	−
III												
Jan. 6 Mar. 1	L. P.	57	♂	Moderately severe chronic interstitial nephritis	Improved	8.0 4.9	80 17	4.8 2.9	0 10	240 170	++	++
April 23 May 21	J. P.	34	♂	Moderately severe chronic diffuse nephritis	Improved	8.3 5.3	72 21	3.2 1.9	25 43	238 145	+++	++
Jan. 15 Jan. 28	W. C.	49	♂	Moderately severe chronic diffuse nephritis	Improved	9.5 2.5	44 19	3.5 1.9	38 52	210 120	++	++
IV												
April 11	E. C.	50	♀	Typical fatal case of chronic interstitial nephritis	Died	22.4	236	16.7	0	210	++	Pus
Mar. 23	T. D.	34	♂	Typical fatal case of chronic interstitial nephritis	Died	15.0	240	20.5	2–3	225	++	+
Jan. 25	S. H.	37	♂	Typical fatal case of chronic interstitial nephritis	Died	14.3	263	22.2	0	220	++	+
April 15	J. W.	34	♂	Typical fatal case of chronic interstitial nephritis	Died	8.7	144	11.0	Trace	225	+	+

From Chace and Myers' article, Jour. Am. Med. Assn., 1916, lxvii, 929.

TESTS FOR ACID INTOXICATION.

Acidosis has been defined as a depletion of the blood and other tissues of the body in fixed bases (Sellards). It may result from the defective oxidation of abnormally formed organic acids as in diabetes, or from the decreased elimination of acid substances which are normally present, as in nephritis. The "alkali reserve" of the serum, by means of which acids are neutralized, is made up of the bicarbonates, small quantities of phosphates, and alkaline protein compounds. Under normal conditions these substances are present in quite constant quantities and reaction is maintained in a state of constant equilibrium. An acid product normally present is carbon dioxide. This is taken up by the serum partly in solution and partly in combination with the carbonates. When it is increased in amount, there is an overstimulation of the respiratory center with a resultant increase in pulmonary ventilation and a greater loss of carbon dioxide so that the reaction of the blood is kept for a time within normal limits, and there is no draft upon the "alkali reserve." With increasing amounts of acid, however, non-volatile acids combine with the carbonates. These cannot be eliminated by increasing pulmonary ventilation and they combine with the carbonates, which are called by Henderson the "first line of defense" and encroach upon the alkali reserve. For a time urinary excretion may serve as a compensatory mechanism since the kidneys have the power of excreting an acid urine from a neutral blood, thus eliminating acid. It has been shown that this mechanism fails in certain forms of nephritis so that the inorganic phosphates of the serum are increased. In acidosis, even the normal amount of carbon dioxide lowers the already depleted alkali reserve. As a consequence the respiratory center is greatly overstimulated, hyperpnea results, and the carbon-dioxide tension of the alveolar air is greatly reduced.

Consideration of the Methods.—Several methods have been proposed for the determination of the degree of acidosis. Chief among these are the determinations of: (1) diminished carbon-dioxide combining power of the blood; (2) diminished carbon-dioxide tension of the alveolar air; (3) increased tolerance to sodium bicarbonate; (4) change in the reaction to phenolphthalein, from alkaline to neutral, of the protein-free filtrate of the blood serum; (5) increase in the hydrogen-ion concentration of the blood; (6) appearance of acetone bodies in the urine; (7) increase in the output of urinary ammonia.

The appearance of acetone bodies in the urine (6) can be expected only in carbohydrate acidosis, and the increase in urinary ammonia (7) is usually not found in nephritic acidosis. It is rather generally felt that the determination of hydrogen-ion concentration of the blood (5) is not a clinical method of great value on account of the obvious difficulties with colorimetric determinations made upon blood-serum or even its dialysate and because it has been shown that no decided change occurs except in advanced cases (Sellards). The change in reaction in a protein-free filtrate has been commended by Sellards, who proposed this test, as being not only of diagnostic value but also of considerable help in regard to therapy. The determination of the CO_2 combining power of the blood (1) and of the carbon-dioxide tension of the alveolar air (2), and of the tolerance of sodium bicarbonate (3) have been of especial clinical value. Of the first two methods the determination of the carbon-dioxide combining power is much more reliable and is carried out without any especial difficulty. Though the determination of the carbon-dioxide tension of the alveolar air is a simple bedside procedure and is of marked clinical value, the information is not as reliable as that obtained by the determination of the CO_2 combining power of the blood. The bicarbonate tolerance test is the simplest of all.

The methods which we have designated as first, fourth, and fifth have been described in the section of the chapter which follows immediately. The technic for the second method has been given in the chapter on miscellaneous methods and the third method, together with a consideration of the acetone bodies and the output of urinary ammonia, will be found in the chapter on urine.

Determination of the Carbon-dioxide Combining Power of the Blood Plasma (method of Van Slyke and Cullen).—*Principle of Method.*—Oxalated blood is obtained by venipuncture, the plasma is separated by centrifugalization and thoroughly saturated with carbon dioxide. The carbon dioxide combines with the fixed alkalies of the plasma to form bicarbonates. The plasma is placed in an especially devised pipette, where the carbon dioxide is liberated by the action of sulphuric acid in the presence of a vacuum. The watery solution is then removed from the pipette and the liberated CO_2 gas is measured over mercury in the calibrated portion. It is evident that the amount of carbon dioxide which will be taken up by the blood and subsequently liberated depends upon the amount of fixed alkali in the plasma, and therefore that the CO_2 combining power will serve as an indication of the alkali reserve.

Apparatus and Reagents Required.—1. Van Slyke's apparatus. This is manufactured by the Emil Greiner Company, 55 Fulton Street, New York City. It should be mounted in a suitable support. The manufacturer ordinarily supplies a frame of wood which is held upon an iron tripod.

2. Mercury sufficient to fill the apparatus and allow a moderate surplus. About 2 kg. will be required.

3. Separatory funnel, holding about 100 times the volume of the plasma to be saturated. It is convenient to have a funnel of 250 cc. capacity.

4. Ostwald pipettes, one of 1 cc. and one of 0.5 cc. capacity.

5. Apparatus for removing moisture from the exhaled air. A glass

bottle should be filled with glass beads and fitted with inlet and outlet tubes as shown in Fig. 58.

6. Apparatus for withdrawing blood by venipuncture.

7. Dropping bottles containing the following:

(*a*) Phenolsulphonephthalein solution, 1 gm. dissolved in 100 cc. of 95 per cent. alcohol.

(*b*) Caprylic alcohol.

(*c*) Sulphuric acid, 5 per cent. solution.

(*d*) Ammonia, 1 per cent. concentration. The ammonia should be freed from carbonate by adding a small amount of barium hydrate. The precipitated barium carbonate can be filtered off, when the excess of barium is precipitated with a little ammonium sulphate.

8. Medicine droppers, beaker containing distilled water, etc.

Obtaining the Blood.—Blood may be obtained by venipuncture from an arm vein. It should be drawn directly into a dry and chemically clean centrifuge tube into which should have been placed enough powdered potassium oxalate to make about 0.5 per cent. of the weight of the amount of blood to be obtained. For withdrawing the blood, either the MacRae needle or the one described by the writer may be used. The technic of venipuncture has been described fully on page 126. It is desirable to avoid stasis of the blood so that the use of a tourniquet should be dispensed with when possible. If indispensable, it should be released as soon as the vein has been entered and a few seconds should be allowed to elapse so that the stagnant blood may pass before the specimen is withdrawn. After the specimen has been obtained, the tube is stoppered with a clean cork and turned on its side and back to the vertical position once or twice to ensure thorough mixing with the oxalate. The blood is then subjected to no further agitation and is centrifugalized in the same tube as soon after withdrawal as possible to separate the serum.

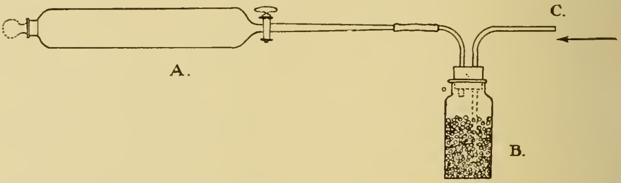

FIG. 58.—Apparatus for saturating plasma with CO_2. *A*, separatory funnel; *B*, bottle containing glass beads; *C*, mouth-piece through which air is expired.

The patient should have been instructed to avoid vigorous muscular exercise for at least an hour before the withdrawal of the specimen.

After centrifugalization, the supernatant clear serum is pipetted off with a capillary pipette. When the determination is to be made immediately, the serum may be placed directly into the separatory funnel when the plasma has been separated at once, immediately put into paraffined tubes and kept on ice, it has been shown that it will retain its carbon-dioxide capacity for over a week without alteration.

Saturation of Plasma with Carbon Dioxide.—The plasma is placed in a separatory funnel (Fig. 58, *A*). This is placed on its side and the contained air displaced by exhaling into it the alveolar air from the operator's lungs. This air is passed through a bottle containing glass beads to free it from moisture (Fig. 58, *B*). The operator should not inspire more deeply than normal and should expire as quickly and completely as possible. The stopper in the funnel should be inserted just before the inspiration is finished, so that air will not be drawn back into the funnel. The stopcock is turned to seal the chamber at both ends, and the funnel is turned end over end for two minutes in order to distribute the plasma in as thin a layer over the inner surface of the funnel as possible. Two minutes are required for saturation. A number of sera may be saturated at one time, using different funnels for each serum, and standing them upright in a rack until ready for the actual determination.

When plenty of serum is available, 3 cc. are saturated in the funnel. This will yield enough for two determinations. When the supply is limited, 0.5 cc. may be used in a determination, saturating the serum available in a 50 cc. funnel. In this case, the total volume of water, serum, and acid used in the determination should not exceed 1.25 cc., instead of 2.5 cc. as prescribed when a 1 cc. quantity of serum is utilized. When a 0.5 cc. quantity is employed, the volume of gas should be multiplied by two before making the corrections.

After saturation is completed, the funnel is stood in an upright position until the serum drains to the bottom of the chamber, from which it may be taken up with the Ostwald pipette of the proper capacity (Fig. 54, *d*). In delivering the serum in cup *b* of the apparatus, it should be allowed to run in under the level of the dilute ammonia in the cup.

Preparation of the Apparatus.—The apparatus (Fig. 59) is completely filled with mercury, including the capillaries *a* and *b* above the cock *e*. The mercury is run into the instrument through the leveling bulb. To fill capillary *b*, the leveling bulb should be in position *1*, the cock *e* should be opened to the position *X'*, and the cock *f* should be placed first in the position *Y* and then in the position *Y'* to allow the mercury to rise first through *c* and then through *d* to fill *A* and partly fill *b* and so that all the air will be forced out. When *b* has been partly filled, cock *e* is turned to position *X* so that some of the mercury may run out through *a* into suitable vessel, filling *A*.

The apparatus should be tested for tightness and freedom from gases. The cock *e* should be closed and the leveling bulb lowered to position *3*, stopcock *f* being open, connecting bulb *A* and smaller bulb *d*. A torricellian vacuum is obtained by allowing the mercury to fall to about the middle of bulb *d*. The leveling bulb is raised quickly to position *1*.

A sharp click will be heard as the mercury strikes the upper stopcock provided that there is not a cushion of air or water between the stop-

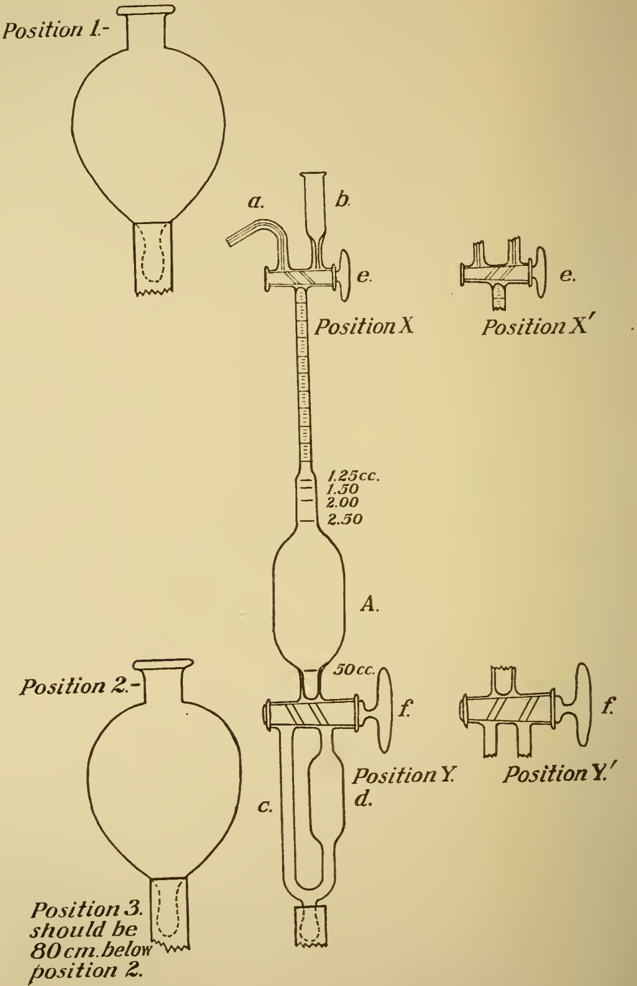

Fig. 59.—Van Slyke's apparatus for determination of CO_2 combining power of plasma. (Figure modified from Van Slyke's.)

cock and the upper surface of the column of mercury. If there be any fluid or air, it must be evacuated by turning cock *e* to position *X* and draining it out through outlet *a*, when the outlet should be closed and the apparatus again tested. Van Slyke has called attention to the fact that before the apparatus has been used it holds measurable amounts of gas which come from the rubber tubing and even from the glass itself. These gases are liberated by the vacuum.

The stopcocks should be lubricated by a suitable stopcock grease (which may be obtained from the manufacturers) and held in place by stout elastic bands or elastic cords made of fine wire spirals.

After using the apparatus, it is not necessary to wash it out before making the next determination. The cup *b* should be washed out with ammonia and the reaction should be tested with phenolphthalein to make certain that all the acid has been neutralized.

When not in use, the mercury should be drained out and the apparatus should be filled with water after thoroughly greasing the cocks. The mercury may be cleaned occasionally by forcing it through clean chamois. To do this a piece of chamois about 12 inches square is held over a large beaker and the mercury is poured into the chamois, which is gathered up like a bag, with the mercury inside. The neck of the sack is twisted tight, the bag is squeezed, and the mercury is forced through the chamois into a large beaker.

The Determination.—The mercury is removed from cup *b* which is washed out with carbonate-free ammonia by running some in and by removing most of it with a medicine dropper. A trace is left and a few drops of water with a drop of phenolphthalein solution are added. If sufficient ammonia be present, the solution will be a distinct red. One cc. of the serum which has been saturated with carbon dioxide is run into the cup from an Ostwald pipette, whose tip should be kept beneath the level of the diluted ammonia water so that there will be no loss of the carbon dioxide into the air. The last of the serum should be ejected from the pipette by keeping the upper end tightly closed with the finger tip while the bulb of the pipette is grasped firmly with the palm of the other hand. The heat is sufficient to drive out the last of the serum. The leveling bulb is lowered to position *2*, the cock *e* is opened to position *X′*, so that the cup *b* and the 50 cc. bulb are in communication, and the serum is drawn into the calibrated upper portion of the 50 cc. chamber leaving just enough to fill the capillary portion of *b*. The cock is closed and in the cup is placed about 0.5 cc. of water, which is drawn into the 50 cc. chamber just as the serum was. This is repeated. Next a drop of caprylic alcohol is drawn in to prevent foaming, and finally 0.5 cc. of 5 per cent. sulphuric acid. It is not necessary that exactly 1 cc. of wash water and 0.5 cc. be taken, but the total volume of fluid drawn into the 50 cc. must not exceed 2.5 cc. as measured on the calibrated stem. Not a single bubble of air should be permitted to gain access with any of the fluids which are admitted. After the acid has been run in, a few drops of mercury are placed in cup *b* to seal the capillary and the acid in the cup is washed out with water.

When the solutions are all in the pipette and the stopcock is closed and sealed off with mercury, the leveling bulb is lowered and hung in position *3*. The cock *f* is opened (position *Y*) so that the mercury in the 50 cc. portion can run into the bulb *d*. The mercury is allowed to run out until the upper surface of the mercury has reached the 50 cc. mark of the chamber *A*, when the cock is closed. The 50 cc. chamber is now sealed above and below. The apparatus is now detached from the stand and the pipette is turned upside down and back for at least fifteen times to ensure obtaining an equilibrium between the 2.5 cc. of water solution and the 47.5 cc. of free space in the apparatus. The apparatus is then replaced in its support with the leveling bulb still in position *3*. The lower stop cock is turned to position *Y* so that the watery solution may be drained into chamber *d* as completely as possible, not allowing any gas to follow it. Of course, it will be impossible to drain the last few drops which will float as a layer on the upper surface of the mercury, but the error caused by the absorption of carbon dioxide by the water will be so small that it may be neglected

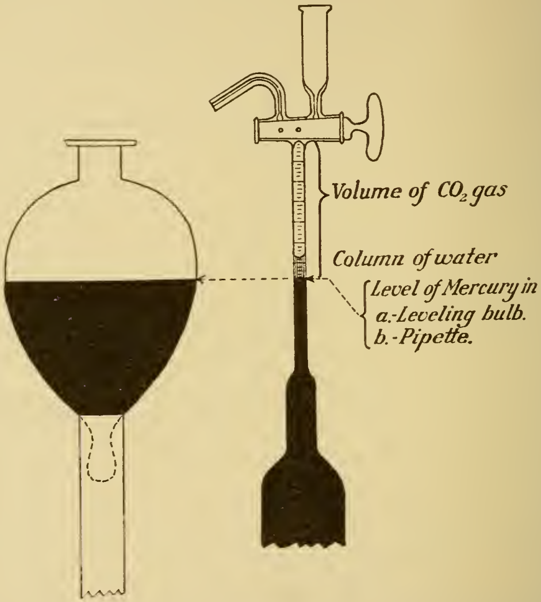

Fig. 60.—Final reading of Van Slyke's apparatus. (After Van Slyke and Cullen.)

provided the reading be made at once. The cock is then closed, the leveling bulb is taken in the left hand, while with the right hand the cock *f* is opened to position *Y′*, to connect *c* with the 50 cc. chamber, so that the mercury will fill up the chamber, a few cc. of water floating on top of the column. Then the mercury bulb is held so that the upper surface will be on the same level as the mercury meniscus in the calibrated portion of the pipette (Fig. 60) and the reading is taken on the scale. This concludes the analysis. The corrections should be made for temperature and barometric pressure.

When the determination has been completed, the lower cock is opened (position *Y′*) so that the 50 cc. chamber and side tube *c* are in communication. The leveling bulb is lowered and most of the mercury is drawn out through *c*. The cock is then turned (position *Y*) so that the watery solution may be re-admitted from *d* to *A*, raising the leveling bulb to position *1*. Cock *e* is opened (position *X*) forcing out the water solution and a little mercury through capillary *A*.

Calculation.—This calculation may be carried out with the aid of the two tables which have been worked out by Van Slyke and Cullen. The volume of gas observed is multiplied by the factor $\frac{B}{760}$. This factor may be ascertained from Table I, when the barometric pressure at which the determination was made is known. For example, if the barometric pressure were 750, the factor $\frac{B}{760}$ is seen to be 0.987. The volume of gas observed is multiplied by this and the resultant figure is located in the first column of Table II, the column headed "Observed volume of gas × $\frac{B}{760}$." The line is carried across horizontally to the column which is headed by the temperature at which the reading was made, and the result is read off. This is expressed in terms of cc. of carbon dioxide bound by 100 cc. of plasma, reduced to 0 degrees, and a barometric pressure of 760 m. Figures for temperatures between those which are given may be calculated.

TABLE I.

For convenience in the calculation the values for the ratio $\frac{\text{barometer}}{760}$ over the range usually encountered are given below.

Barometer.	$\frac{\text{Barometer.}}{760}$	Barometer.	$\frac{\text{Barometer.}}{760}$
732	0.961	756	0.995
734	0.966	758	0.997
736	0.967	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.011
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

TABLE II.—TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA.

Observed vol. gas $\times \frac{B}{760}$	Cc. of CO_2 reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. of plasma.				Observed vol. gas $\times \frac{B}{760}$	Cc. of CO_2 reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. of plasma.			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

Example.—If the observed volume of gas were 0.50, the barometric pressure 750 mm., and the temperature 20 degrees, the figure .50 would be multiplied by the factor $\frac{750}{760}$ which in consulting Table I, is found to be 0.987, as explained above. 0.50 times 0.987 is equal to

0.4935. The nearest figure to this found in Table II is 0.49. Carrying the line across horizontally to the column headed by the temperature 20 degrees we find the figure 37.6. The difference between this figure and the one just beneath is 0.9. We take one-third of this to allow for the fraction expressed in the third and fourth decimal places of the figure 0.4935, and add it to 37.6, making our final result 37.9, which expresses the number of cc. of carbon dioxide bound as bicarbonate by 100 cc. of plasma, reduced to 0 degrees and 760 mm. pressure.

Remarks.—On account of the added complication of making these computations, some workers recommend subtracting from the observed volume of gas for the purpose of correction the arbitrary figure 0.12. In the above example, for instance, 0.12 taken from the observed volume of gas, 0.50, would give the result 0.38, which closely approximates that obtained in the example.

Normal Findings.—The bicarbonate carbon dioxide absorbed and yielded by the plasma amount to 0.53 to 0.75 cc. per cc. of plasma, or 53 to 75 cc. per 100 cc. of plasma (Van Slyke and Cullen), when the blood has been withdrawn in the manner described. In acidosis, carbon dioxide capacity falls far below normal.

Determination of the Reaction to Phenolphthalein of the Protein-free Blood-serum (method of Sellards).—*Reagents Required.*—1. Absolute ethyl alcohol. The selection of alcohol which will be neutral is of great importance. Sellards states that it is difficult to obtain absolute alcohol of neutral reaction but that it is comparatively easy to obtain 95 per cent. alcohol free from any alkali, and that preliminary tests do not show that this amount of water is disadvantageous. The alcohol should be tested. The test for alkalinity is performed by mixing 50 cc. of the alcohol to be tested with a drop of phenolphthalein and evaporating this to dryness over a water-bath. The residue should be colorless and should remain colorless on the addition of a drop of water. To test, 50 cc. are mixed with 0.1 cc. of one-hundredth normal sodium hydrate and evaporated to dryness with a drop of phenolphthalein. The residue should turn red on drying or after a drop of water is added.

Procedure.—One cc. of serum is added drop by drop to 25 cc. of tested alcohol in a test-tube. The proteins are precipitated. They are filtered off through perfectly dry filter paper in a dry funnel, taking all precautions to prevent the addition of any water. The precipitate is not washed but is evaporated to dryness over a water-bath after the addition of a few drops of phenolphthalein.

Interpretation of Results.—According to Sellards, the alcoholic filtrates from all normal sera soon turn red and on drying, the residues, if kept hot, retain their color for several hours. With early acidosis where there was a deficit of from 20 to 30 grams of sodium bicarbonate (see Sellards' bicarbonate deficiency test under Urine, page 276), the alcoholic filtrate must become very much concentrated before the red color appears and the red color usually disappears soon. With more advanced depletion of bicarbonates, corresponding to a deficiency of 75 to 100 gm., no red color appears at any stage of evaporation, but the residue becomes pink or red on adding water. With a well-marked acidosis corresponding to a deficit of 150 gm. of bicarbonate, the residue remains colorless even on adding water.

Determination of the Alkali-reserve of the Blood Plasma (method of Marriott).—*Principle.*—Blood serum is dialyzed against salt solution to remove proteins and coloring matters which would interfere with the exact matching of the indicators. The carbon dioxide is then removed from the dialysate by aëration so as to leave the unneutralized alkali reserve and then the hydrogen-ion concentration of the dialysate is determined by means of an indicator, phenolsulphonephthalein, using as standards for comparison phosphate solutions of varying hydrogen-ion concentration. Marriott states that the results compare closely with those obtained by the electrical method.

Apparatus and Reagents Required.—

1. Set of tubes containing standard phosphate mixtures.
2. Solution of phenolsulphonephthalein in 0.8 per cent. salt solution.
3. Collodion sacs.
4. Pipette to measure 0.5 cc.
5. Small test-tubes for aërating and dialyzing.
6. Atomizer bulb.
7. Glass tube or pipette drawn out to a fine capillary point.
8. Box for color comparison, with ground glass background, on principle similar to Sahli hemoglobinometer.

Preparation of Reagents. Phosphate Mixtures. (*a*) *One-fifteenth Molecular Acid or Primary Potassium Phosphate* (KH_2PO_4).—The pure re-crystallized salt should be used. 9.078 gm. are dissolved in freshly distilled water. To this are added 200 cc. of 0.01 per cent. aqueous solution phenolsulphonephthalein and the volume is brought up to one liter with distilled water.

(*b*) *One-fifteenth Molecular Secondary Alkaline Sodium Phosphate.*—The pure, recrystallized salt ($Na_2HPO_4.H_2O$) is exposed to the air, protected from dust, from ten days to two weeks. Ten molecules of water are given off and a salt of this formula is obtained, $Na_2PO_4.2H_2O$. Of this salt, 11.876 gm. are dissolved in distilled water, 200 cc. of 0.01 per cent. aqueous solution phenolsulphonephthalein are added, and the whole made up to one liter with distilled water. The same amount of indicator must be added to the two solutions and to the salt solution which is hereinafter described. Mixtures are made according to the amounts given in ccs. in the table below to give differing hydrogen-ion concentrations. After the solutions have been prepared, a crystal of thymol is placed in each flask to prevent the growth of moulds. Small test-tubes about 8 mm. bore by 100 mm. are partially filled with the solutions. The glass should be of a type which does not give off alkali readily, such as Non-sol, Pyrex, or Jena. The upper ends of the tubes should be sealed in the blow-pipe. They should be kept in a dark place, under which condition they retain their colors for some time.

PROPORTIONS IN WHICH PHOSPHATE SOLUTIONS ARE MIXED.

Hydrogen-ion concentration . .	7.0	7.2	7.4	7.6	7.8	8.0	8.2	8.4	8.6
Primary potassium phosphate, cc.	37.0	27.0	19.0	13.2	8.8	5.6	3.2	2.0	1.0
Secondary sodium phosphate, cc.	63.0	73 0	81.0	86.8	91.2	94.4	96.8	98.0	99.0

The terminology which is employed in discussing the question of acidosis is confusing to one unaccustomed to the physico-chemical methods of expression. We can do no better than to quote *verbatim* the concise explanation given by Levy, Rowntree and Marriott: "A solution is acid when it contains an excess of hydrogen over hydroxyl ions, neutral when hydrogen and hydroxyl ions are in equal numbers, and alkaline when hydroxyl ions predominate. An acid of 'normal' strength contains, in 1 liter, 1 gram of hydrogen capable of forming ions, and its strength may be expressed as 1 N. Diluting such a solution ten times, we would have a $\frac{1}{10}$ N or a solution containing $\frac{1}{10}$ gram of actual or potential hydrogen ions to the liter. Continuing the process of dilution until $\frac{1}{10000000}$ normal acid is obtained, we would have in such a solution $\frac{1}{10000000}$ gram of hydrogen ions. Pure water, however, dissociates to form hydrogen ions, and at 20° C. contains approximately $\frac{1}{10000000}$ of hydrogen ions to the liter and an equivalent amount of hydroxyl ions. That is to say, pure water, our standard of neutrality, is $\frac{1}{10000000}$ N acid and also $\frac{1}{10000000}$ alkaline. To avoid writing large figures it is customary to use the logarithmic notation and to express $\frac{1}{10000000}$ N, as 10^{-7} N or, more conveniently, as suggested by Sörensen, to drop the 10 and the minus sign and say pH7. If we have less than $\frac{1}{10000000}$ gram of hydrogen ions to the liter, the solution is less acid than water, that is, is alkaline—so, pH8 means actually $\frac{1}{10000000}$ N alkali. The higher the exponent the more alkaline, or what is saying the same thing, the less acid is the solution.

To sum up:

pH1 = N/10 acid.
pH6 = N/1,000,000 acid.
pH7 = Neutrality.
pH8 = N/1,000,000 alkali.
pH14 = N/10 alkali.

The reaction of the blood serum varies approximately between pH7 and pH8, the neutral point being reached only in severe uncompensated acidosis, and a reaction of pH8 being attained perhaps only after the administration of alkalies."

Preparation of Sacks.—Anthony's negative cotton is used. This is manufactured by the Ansco Company, Birmingham, N. Y. It contains about 30 per cent. of water. This must be poured off and the celloidin rinsed in two or three changes of 95 per cent. alcohol before being dissolved. One ounce is then dissolved in 500 cc. of a mixture of equal parts of ether and 95 per cent. alcohol. The solution should be allowed to stand for a week to permit the impurities to settle.

In making the sacks, a small test-tube about 50 mm. long by 6 mm. bore with a flared mouth is filled with the celloidin solution. It is poured out of the tube slowly, rotating the tube so that the inside will be coated with a thin film of celloidin. The tube is then held in the upright condition so that it may drain for ten minutes, or until it ceases to give off the odor of ether. When the sacks are dried too long they become hard and brittle, while sacks that have not been dried long enough become white and cloudy when water is added.

When the sacks have dried for a suitable length of time they are filled with water and removed from the tubes by running a knife blade around the edge at the top of the test-tube to loosen them, running water between the sack and the tube. The sacks are preserved in water.

Preparation of Salt Solution.—Eight gm. of chemically pure sodium chloride are dissolved in a small quantity of distilled water. 220 cc. of 0.01 per cent. aqueous solution of phenolsulphonephthalein are added and then enough water to bring the total volume to one liter. Marriott recommends making the concentration of the indicator stronger in the salt solution than in the standard phosphate mixtures to allow for loss during the process of dialysis. It is important that the solution contains no free alkali or any acid except carbonic acid. A portion of the solution should be tested by boiling it in a Pyrex glass test-tube to expel carbonic acid. This is cooled quickly and compared with the standards. It should match the pH7 tube. Should the glass of the test-tube give off alkali, the solution will be colored a faint pink. Should the reaction of the solution differ from this, it may be corrected by adding a few drops of very dilute acid or alkali to the entire bulk of the solution. The salt solution must be kept in a flask of Non-sol or Pyrex glass, or in a flask of ordinary glass whose inner surface has been coated with a thin film of paraffin.

Procedure for the Determination.—The procedure should be carried out in a room free from acid or ammonia vapors. While either separated serum, the plasma from oxalated blood, or whole blood may be employed, serum separated from the clot is to be preferred since the introduction of oxalate may introduce an error. The serum should be separated by centrifugalization as soon as possible after the blood has been obtained. Hemolysis should be guarded against.

One-half cc. of clear serum is transferred with the pipette to one of the sacks, which should have been washed inside and out with the salt solution. In a test-tube about 8 mm. in bore and 50 mm. long is placed 2 cc. of the salt solution containing the indicator. The level of the fluid on the inside of the sack should not exceed that on the outside. After seven minutes have elapsed the sack is withdrawn and the dialysate (salt solution) is transferred to a test-tube 100 to 140 mm. long, having the same bore as those containing the standard phosphate solutions. The atomizer bulb is connected with a piece of glass tubing the end of which has been drawn out into a fine capillary. The capillary end is placed in the tube of dialysate and a current of air is blown through to get rid of carbon dioxide. The force of the air-current should not be strong enough to cause the liquid to bubble out of the tube. After the air-current has been allowed to pass for three minutes, the color of the tubes is compared with that of the standards. When the unknown does not exactly match either of two tubes but appears to fall between them in depth of color, the reading should be interpolated.

Interpretation of Results.—Marriott states that with normal individuals the alkali reserve (R) was found to be between 8.55 and 8.45, when the subjects were on a mixed diet. After a fast of sixteen hours, a normal adult's serum gave a reading of 8.35. The sera from infants gave somewhat lower readings, a value of 8.3 being encountered occasionally. He felt that values of 8.4 to 8.5 corresponded to alveolar carbon dioxide tensions of 38 to 45 mm. Values between 8.0 and 8.3 correspond to alveolar carbon dioxide tensions of 28 to 35 mm. and indicate a moderate degree of acidosis, while values as low as 7.7, corresponding to an alveolar carbon dioxide tension of 20 mm. were found in severe acidosis and indicated that the individual was in grave danger.

Significance of the Results of Blood Tests for Acidosis.—Two tests have been applied to the blood for the detection of acidosis:

1. Determination of the carbonate content of the whole blood or plasma—the so-called alkaline reserve.

2. Determination of the hydrogen-ion concentration (pH.) of blood. The latter merits little consideration because the results obtained by its use show little or no deviation from the normal except in moribund cases. Indeed it appears very likely from animal experimentation and from theoretical considerations that any sustained elevation in the actual (pH) of the blood is incompatible with life.

Since the bicarbonate of the body fluids represents the most important alkaline reserve of the tissues, it is apparent that this first line of defence as it were will show deviations from the normal, in conditions of acidosis. It is well established that the blood bicarbonate is depleted in acidotic conditions caused by fixed acids. As examples we may refer to the production of unoxidized fatty acids, like β-oxybutyric and aceto-acetic in diabetes, and the improper elimination of acids produced in normal metabolism as happens in some cases of nephritis.[1]

When, however, an accumulation of carbonic acid occurs in the body the bicarbonate of the blood is elevated above the normal. This has been demonstrated by R. W. Scott in animals made to breathe CO_2 and recently he has reported a similar condition in patients with chronic pulmonary emphysema of the "large lunged type."[2]

Determination of Chlorides of Blood.—The methods which are given may be used for determining the chloride content of either plasma or whole blood. The content of whole blood normally amounts to 0.45 to 0.50 per cent.; figures for the plasma are about 0.12 per cent. higher. While it would seem more logical to determine the content of the plasma, which bathes the body tissues, the examination of whole blood is really preferable, since unless the plasma is separated from the cells almost immediately, its chlorides increase at the expense of the corpuscles due to passage of carbon dioxide from plasma to the corpuscles or its escape into the air.

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[1] A recent discussion of acidosis in nephritis with bibliography is given by Chase and Meyers, Jour. Am. Med. Assn., 1920, lxxiv, 641.

[2] Proc. Soc. Exp. Biol. and Med., 1919, xvii, p. 18.

Discussion of Methods.—The two methods given are equally accurate. The method of Myers and Short has certain points of advantage over Austin and Van Slyke's iodometric method. The reagents are dilutions of those used for the determination of urinary chlorides by the Volhard-Harvey method; they are more stable; and the procedures are carried out more rapidly. Furthermore, if methods be used for determining blood sugar and creatinine which employ picric acid to precipitate proteins, a portion of the same protein-free filtrate may be used for the chloride determination.

Method of Myers and Short.—Principle.—The proteins are precipitated with picric acid as in the methods for the determination of blood sugar and creatinine. After separation of the precipitate, the protein-free filtrate is used for the determination of chlorides by the Volhard-Harvey method, the principle of which is described in the method for determination of urinary chlorides.

Reagents Required.—

1. Silver nitrate solution, 2.904 gm. to 1000 cc. of water. One cc. is equivalent to 1 mg. of sodium chloride. This may be prepared by making a one to ten dilution of the solution used by determining urinary chlorides by the Volhard-Harvey method.

2. Acid ferric alum indicator solution prepared by dissolving 100 gm. of crystalline ferric ammonium sulphate in 100 cc. of 25 per cent. nitric acid and adding 4 volumes of distilled water. This is one-fifth the strength of the solution used for the urine determination.

3. Ammonium thiocyanate of such strength that 2 cc. are equivalent to 1 cc. of the silver solution (No. 1). It contains approximately 0.65 gm. of the thiocyanate in 1000 cc., but must be checked by titration against the silver nitrate solution.

4. Picric acid crystals, C. P.

Procedure.—In a 50 cc. centrifuge tube are placed 3 cc. of whole blood (or plasma) and 27 cc. of distilled water. About 0.5 gm. of dry picric acid are added and the mixture is stirred until protein precipitation is complete, when a bright yellow "mustard" color appears. The tube is centrifugalized and the supernatant clear fluid is decanted into a dry beaker. Should any particles remain the filtrate is poured through a dry filter. 20 cc. of the filtrate are transferred with a pipette to a clean dry centrifuge tube when 20 cc. of the standard silver nitrate solution and 10 cc. of the acid ferric alum indicator are added. After stirring, the precipitated silver chloride is thrown down by centrifugalization and the clear supernatant fluid decanted into a dry beaker. Duplicate portions of 20 cc. each are measured into porcelain evaporating dishes with pipettes. Titration is carried out by adding the ammonium thiocyanate solution until the first permanent tinge of brown is obtained. While the end-point is definite, some experience may be required before it is recognized invariably. Passing the end-point by one drop introduces an error of 0.5 per cent. in estimating the chlorides in 100 cc. of blood.

If desired, the 1:5 picric acid filtrate may be used for sugar and creatinine. In this case 5 cc. (the equivalent of 1 cc. of blood) are taken adding 10 cc. of the standard silver solution and 10 cc. of a diluted acid ferric alum indicator solution (the solution described above as No. 2 diluted with an equal quantity of water). After precipitation of the chlorides and centrifugalization, a single 20 cc. portion of filtrate is titrated as described above. Myers and Short have shown that the results above with 1:5 and 1:10 filtrates are practically identical.

Calculation.—This is made with the formula:

$$10 - \left(\frac{\text{titer}}{2} \times \frac{5}{4}\right) \times 100 = \text{mg. of sodium chloride in 1000 cc. of blood (or plasma).}$$

Austin and Van Slyke's Method for Determining Blood Chlorides.—Principle.—The proteins are precipitated from blood serum or an oxalated specimen of whole blood with picric acid. The coagulum is removed and the chlorides in the protein-free filtrate are precipitated in the presence of nitric acid by a standard silver nitrate solution. The amount of uncombined silver nitrate is determined by titration with a standard potassium iodide solution in the presence of nitrous acid and starch. The first drop of iodine in excess of the silver present is changed to free iodine and gives the blue starch-iodine color reaction. The optimum acidity for the end-point is secured by the addition to the starch solution of trisodium citrate. Knowing the uncombined silver, the combined silver is readily found, and the amount of chlorides determined from this.

Reagents Required.—

Solution 1.—An acid solution of silver nitrate, 1 cc. of which is equivalent to 2 mg. of sodium chloride.

Silver nitrate	5.812 gm.
Nitric acid (sp. gr. 1.42)	250.000 cc.
Distilled water to	1000.000 cc.

McLean and Van Slyke state that in place of silver nitrate, metallic silver may be employed. This may be obtained in a high state of purity. In this case, 3.688 gm. of silver are dissolved in the prescribed amount of nitric acid and the total volume brought to 1000 cc. with distilled water. It may be convenient to make up a stock solution of silver nitrate of ten-fold this concentration and to prepare the test solution from this by dilution. The strength of the solution may be checked by titration against a known solution of sodium chloride, as directed on page 250.

Solution 2 (Van Slyke and Donleavy).—A solution of potassium iodide, 1 cc. of which is equivalent to 0.8 mg. of sodium chloride.

Potassium iodide	2.4 gm.
Distilled water to	1000.0 cc.

This solution must be standardized against the silver solution and diluted to the extent indicated by the preliminary titration. Five cc. of the silver solution (1) measured with a calibrated pipette are added to 5 cc. of the starch solution (3) and 5 cc. of water. The iodine solution is run in from a burette until the blue end-point is obtained. It should require exactly 12.65 cc. of the iodine solution to secure this end-point with 5 cc. of the silver solution, 12.50 being required to precipitate the silver and 0.15 cc. additional to give the end-point. The iodine solution must be diluted to secure this concentration.

Solution 3.—This is a solution for use in the final titration containing sodium citrate, sodium nitrite, and starch, which substances respectively regulate the acidity, provide an oxidizing agent for the iodine, and serve as an indicator.

Sodium citrate, crystalline ($Na_3C_6H_5O_7.5\frac{1}{2}H_2O$)	446.0 gm.
Sodium nitrite ($NaNO_2$)	20.0 "
Soluble starch	2.5 "
Distilled water to	1000.0 cc.

The starch should be dissolved by boiling in about 500 cc. of water. The citrate and nitrite are then added, heating the mixture until all are dissolved. While still hot the solution is filtered through cotton, the filter washed with hot water; the filtrate allowed to cool; and the volume made up to 1000 cc. Filtration serves to remove insoluble substances, chiefly occurring in the nitrite. The solution keeps indefinitely, and though it becomes cloudy, its efficacy is not impaired.

Procedure.—In a 60 cc. volumetric flask are placed 3 cc. blood-serum or whole blood with 15 cc. of water. The pipette is rinsed by drawing up and ejecting some of the water two or three times into the pipette. Only a pipette should be used which has been carefully calibrated. Thirty cc. of a saturated solution of picric acid should be added and sufficient water to bring the total volume to 60 cc. When a volumetric flask is not available, the water (27 cc. in all) and the picric acid solution may be added from burettes. The contents of the flask are mixed and filtered after ten minutes' standing. To 40 cc. of the filtrate are added 10 cc. of the standard silver solution (Solution 1) with two drops of caprylic alcohol. The solution should be thoroughly mixed and allowed to stand over night when the clear supernatant fluid is decanted through a small filter paper. Duplicate portions of the filtrate of 20 cc. each are placed in 100 cc. Erlenmyer flasks with a calibrated pipette. Four cc. of the starch-citrate solution (Solution 3) are added to each flask, and the standard iodide solution (Solution 2) is run in from a burette until a permanent blue end-point is obtained. Only a permanent and unmistakable blue is taken as the end-point.

Calculation.—The formula for calculation has been simplified by Van Slyke and Donleavy to the following:

$$\left.\begin{array}{l}\text{Mg. of sodium chloride per cc. of blood, or}\\ \text{Gm. of sodium chloride per liter of blood}\end{array}\right\} = 10.15 - \text{cc. KI solution.}$$

In other words, the number of cc. of KI solution used to secure the end-point is subtracted from 10.15 and the result represents the number of mg. of sodium chloride per cc. of blood. The figure 10.15 is used instead of 10 because of the fact that an excess of 0.15 cc. of iodine solution is necessary to produce the end-point.

Caution.—Attention is directed by Van Slyke and Donleavy to the necessity for calibrating all pipettes and volumetric glassware used in this determination, because there is not much difference between the significant figure and the normal figure of blood chlorides. This may be done by weighing at a temperature of 20° C. the pipettes, etc., dry and when filled with water, and by noting the difference in the weights.

Findings.—Normally, the chloride content of whole blood is from 0.45 to 0.50 per cent. and of plasma from 0.57 to 0.62 per cent. High chloride contents have been found in nephritis, especially in parenchymatous nephritis, certain cardiac conditions, anemia, and in some cases of malignancy, while low values are observed in fevers, diabetes, and pneumonia. The excretion of chlorides and nitrogen appear to be more or less independent functions, since in parenchymatous nephritis the excretion of chlorides is much more impaired than that of nitrogen while with interstitial nephritis, the reverse is true.

Determination of Oxygen of the Blood.—The oxygen content of blood is of significance in certain clinical conditions, notably in cardiac decompensation. It is stated that in normal individuals, the oxygen content of venous blood is about 13.5 volume per cent., or two-thirds of the total amount in the blood when it is saturated. Lundsgaard recommends the following method for determination, involving two steps, first, the drawing of a sample of venous blood, and second, the determination of its oxygen content. Oxygen is determined by Van Slyke's technic, which has been described previously (see page 20). The apparatus for drawing blood consists of a very sharp needle connected by means of a piece of rubber tubing 3 or 4 cm. long to a glass pipette of about 0.5 cm. bore. The pipette and rubber tubing should contain sodium oxalate crystals. These are introduced by wetting the interior with a saturated solution of oxalate, which is dried with a current of air. The upper end of the pipette is stuffed with cotton and placed in the mouth when the needle is thrust into the vein in the proximal direction. Only gentle suction will be needed in drawing the blood. Should air be aspirated through the blood, the specimen should be discarded and a new pipette used, keeping the needle and rubber connection *in situ.* When from 6 to 12 cc. of blood have been secured, the upper end of the pipette is closed with the finger and the needle is withdrawn. The rubber tubing is disconnected and the blood is discharged into a cylinder of about 2 cm. diameter (with a few crystals of sodium oxalate in the bottom) under a layer of white mineral oil about 2 cm. deep. The last 0.5 or 1.0 cc. of blood is discarded on account of oxidation. The blood in the cylinder is then mixed with the oxalate by stirring with a glass rod. From the cylinder, 2 cc. samples are introduced into Van Slyke's apparatus beneath a layer of evacuated dilute ammonia. The blood should be stirred before removing each sample to secure homogeneity. Only fresh specimens should be used.

The oxygen content and the oxygen combining power are determined. The latter may be calculated from the determination of hemoglobin by Palmer's method. The difference may be found between the oxygen in venous blood and the total oxygen combining power of the hemoglobin. This difference is termed *oxygen unsaturation* of the venous blood by Lundsgaard, who found that in normal individuals the values ranged from 2.5 to 8 volume per cent., while with uncompensated heart disease, the values were from 9.7 to 15.2 volume per cent. Since normal figures were found only in a stage of full compensation or in decompensation with rapidly lessening symptoms while figures above the upper normal extreme were met with (1) during decompensation, (2) during compensation just before clinical symptoms of decompensations developed, or (3) at times in patients with auricular fibrillations in complete and stable compensation, he feels that the determination of oxygen unsaturation afforded an objective criterion of the positive effect of digitalis therapy.

CHAPTER II.

EXAMINATION OF URINE.

Introductory.—The examination of urine yields much information of great value to the clinician. The writer has often felt, however, that the question of urinary examinations has been more strongly emphasized by many writers and by occasional clinicians than was justified by the value of the information received. Certain tests are described and carried out whose diagnostic value is questionable and much of the quantitative work which is done is valueless, even from the standpoint of routine records, because no attention has been paid to the intake of protein, carbohydrates, and chlorides. Indeed the use of quantitative methods in urinalysis is irrational unless at least the twenty-four-hour output be obtained (a simple consideration frequently overlooked), and unless the diet be taken into consideration. The examination of the urine for clinical purposes can be and should be reduced to extremely simple terms. An analysis of urine should be part of the routine examination of the patient and may be made by the clinician or a trained assistant with modest equipment and small expenditure of time. When indicated, bacteriological examinations and quantitative determinations may be referred to a well-equipped laboratory, though there is no reason why a clinician with a modern training should not be able to carry out readily any useful urinary examination.

In this section, we shall attempt to give an outline for a simple routine examination of the urine, to indicate tests of real clinical significance, to recount the clinical significance of the findings, and to describe the technic of quantitative tests which may be desired in exceptional cases.

ROUTINE EXAMINATION.

A routine examination of the urine should include the following:

1. Color.
2. Appearance.
3. Reaction.
4. Specific gravity.
5. Qualitative test for albumin.
6. Qualitative test for sugar.
7. Microscopical examination of sediment.

In case quantitative determinations are desired, a twenty-four-hour specimen should be obtained.

Apparatus Required.—The laboratory equipment which is needed for the routine examination of the urine is included in the minimal equipment for the clinical laboratory. (See Appendix.)

Daily Amount.—It should be remembered that a knowledge of the amount excreted in twenty-four hours is of the greatest importance in the clinical management of the various forms of nephritis, cardiac decompensation, and diabetes mellitus. This simple observation, judiciously interpreted, may give vastly greater information than laborious quantitative determinations of various constituents. When there is a complete suppression of urine, the condition is spoken of as *anuria* or *anuresis;* when the urine is greatly diminished in amount, the term *oliguria* or *oliguresis* is applied. *Polyuria* refers to an increased quantity.

Order of Procedure.—In order to conserve a specimen when only a small quantity is furnished, a definite line of procedure should be followed. It is possible to make a complete qualitative examination with 50 cc. or even less if economy be exercised. First, take the specific gravity, then start a portion in the centrifuge, next take the reaction, perform the test for albumin and sugar, and finally examine the sediment microscopically. It is better to know one or two tests thoroughly and to know their limitations than to have a superficial acquaintance with many methods. The following are recommended because of their simplicity and their reliability.

Appearance of Specimen.—This should give a clue to special tests. A *dark brown color* suggests the presence of bile; *reddish or chocolate-colored urine,* the desirability of chemical and microscopical tests for blood, though scanty and febrile urines are dark because of concentration. *Turbidity* may be due to (1) bacteria, in which case no clearing will be seen on heating or by the addition of acid; (2) phosphates which dissolve on the addition of acids; (3) urates, which may be white or red. In the latter instance the color may deceive the patient into thinking that blood is present, but cloudiness disappears promptly on heating; (4) pus, which may be centrifugalized to the bottom of the tube but dissolves neither with heat nor with dilute acid and is, of course, readily recognized on microscopic examination.

Mucous shreds should be noted when present. They are white bodies which are readily visible macroscopically, looking like small curled threads, occasionally assuming an almost spiral form. When teased out upon a slide and stained with methylene blue they will be found to consist of an albuminous matrix with embedded epithelial cells, polymorphonuclear leukocytes and bacteria, and a variable amount of mucus. Frequently gonococci may be demonstrated, since they occasionally persist for years in the urine of apparently cured cases. They are sometimes referred to as "gonorrheal shreds" or "clap shreds."

Preservation of Specimens.—If possible the specimen should be examined shortly after it has been voided. When prompt examination is impossible and it is necessary to keep the specimen, a preservative should be added, such as 2 drops of 40 per cent. formalin to the liter of urine. In making chemical analysis, however, it should be remembered that formalin may reduce Fehling's solution.

Specific Gravity.—The float (Fig. 61, *a*) should rest in the cylinder of urine without touching the sides of the cylinder. The reading should be taken from the bottom of the meniscus. If foam is bothersome, a drop or so of ether may be added to cause its subsidence. A urinometer has been placed on the market recently which makes use of the principle of the hydrometer commonly used in testing storage batteries (Fig. 61, *b*). With this the urine may be aspirated into a cylinder containing the hydrometer float. It should prove especially useful where many specimens are to be examined, as in a hospital laboratory. It is needless to say that it should be rinsed carefully before and after each test.

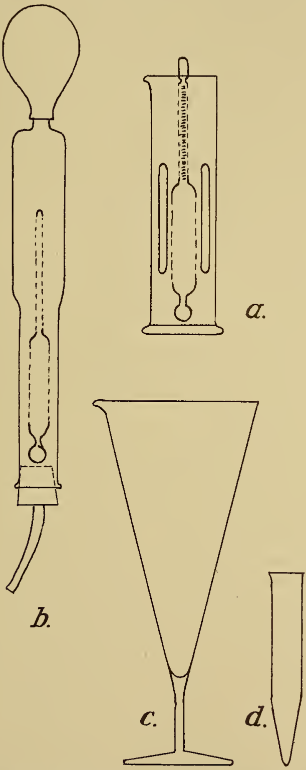


Fig. 61.—Apparatus used in routine urinalysis. *a*, urinometer; *b*, new type of urinometer; *c*, conical sediment glass; *d*, centrifuge tube.

Qualitative Tests for Protein Bodies.—The following tests react with both albumin and globulin, which we do not attempt to consider separately in clinical work. These tests are preferred because of their simplicity and because of the ease with which the reagents can be obtained. The test with heat and acetic acid is the more delicate, sufficiently so for all clinical purposes. We do not feel that more delicate tests than these are either necessary or desirable, since the extremely faint traces of albumin which they reveal are of doubtful clinical significance.

Heat and Acetic Acid Test.—Fill a clean test-tube almost to the top with clear urine. If turbid the urine should be filtered, or the supernatant clear urine in the centrifuge tube may be used. When clear urine can be obtained in neither way, a small amount of Kieselguhr should be shaken up with the urine, which may then be filtered. Heat the top layer in the test-tube to the boiling point. The bottom portion, should not be heated since it serves as a contrast. If a cloud occur add a *few* drops of 5 per cent. acetic acid. Stronger acid or an excess of acid should be avoided, since either will cause the albumin to go into solution. This urine is heated again. If the cloudiness remain after the addition of acid, it is due to albumin or nucleoprotein, but if it disappear, it is due to phosphates. The reason for the appearance of a precipitate of phosphates when previously clear urines are heated is that carbon dioxide is liberated by heating, and the reaction of the urine is made more alkaline.

Heller's Nitric Acid Ring Test.—Put about 3 cc. of nitric acid (C. P.) in the bottom of a clean test-tube and allow 5 cc. layer of urine to run over the acid from a pipette. This should be done slowly and carefully so that there will be distinct layering of the two fluids and so that mixing does not occur (Fig. 62). A white layer at the junction indicates the presence of albumin. A similar layer may be given by certain drugs. A white ring is occasionally given by uric acid or the urates. This ring usually has a more diffuse upper border and occurs at times *above* the line of contact. As a confirmatory measure the urine may be diluted with 3 or 4 parts of water and the test repeated with nitric acid. Uric acid or the urate will not give a ring when the urine has been so diluted.

Sulphosalicylic Acid Test.—The urine is placed in a test-tube and a drop or so of a 20 per cent. solution of sulphosalicylic acid is added. A white precipitate is seen when albumin is present. The urine may be layered over a small quantity of the acid as was directed for Heller's test. This reagent does not give a precipitate with the resins or with uric acid. Albumose gives a precipitate which clears up on heating and reappears on cooling.

Magnesium Sulphate Test.—Put 3 or 4 cc. of acid-magnesium sulphate mixture in a test-tube and run the urine from a pipette to form a layer over the reagent. A white ring will be formed at the junction when albumin is present. The reagent is prepared by adding 1 part of nitric acid (C. P.) to 3 parts of a saturated solution of magnesium sulphate.

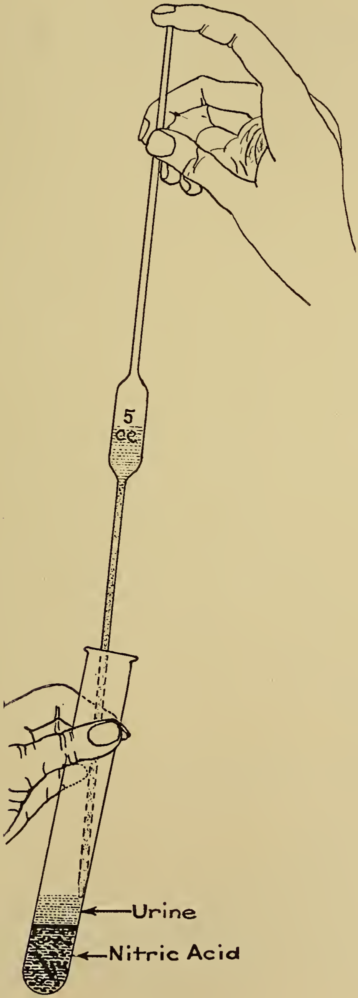


Fig. 62.—Heller's nitric acid test for albumin.

Record of Albumin Findings.—In recording findings, it is advisable to adopt a fixed terminology for the sake of comparison. The following is suggested. It is based on the density of the cloud obtained with the heat and acetic acid test. FPT=faintest possible trace, meaning the least cloud which is visible when the tube is held against a dark background. FT=faint trace. The cloud is slight but readily visible without a dark background. T=trace. There is a distinct cloud, which is sufficiently dense to render the tube not transparent. HT=heavy trace. The cloud is dense and shows flakes. In such cases, albumin is present in a measurable quantity.

Significance of Albuminuria.—In normal individuals, plasma proteins do not escape into the urine in quantities which would render them appreciable by the methods usually employed. They may filter through under certain abnormal conditions due to change in the permeability of the kidney brought about by inflammation or asphyxia, the condition being referred to as renal albuminuria. In addition, protein may be found in the urine without renal damage due to the admixture of inflammatory exudates in other parts of the urinary tract, the so-called *accidental albuminurias.*

Dietetic albuminurias are recognized and are seen after the ingestion of abnormally large amounts of protein.

Renal albuminuria is occasionally seen in normal persons after mental strain, severe bodily exertion, cold baths, and in cases of so-called functional albuminuria, especially in lordotic or *orthostatic albuminuria.* Such cases are always to be regarded with suspicion and should not be dismissed until carefully studied to ascertain the underlying cause. Even when albuminuria is apparently functional, in occasional cases definite evidence of kidney damage has been found later, pointing to the fact that so-called physiological albuminuria occurs only where there is a slight insufficiency of the kidney.

Albuminuria of renal origin may be seen in the anemias and leukemias, in chronic passive congestion of the kidneys, as in uncompensated heart disease, in febrile conditions, in poisoning by certain drugs, notably by ether, mercury, cantharides, lead, arsenic, etc. In these cases, the kidney tissue may be damaged, either permanently or temporarily, or the lesion may be a simple passive congestion, and may clear up when the underlying cause is removed. The true nephritides give varying findings. In acute nephritis albumin is usually present in large amount, and diminishes as the inflammatory process subsides. In chronic nephritis of the interstitial or arteriosclerotic type, the amount is frequently small and repeated examinations may be necessary to demonstrate even faint traces, whereas with so-called parenchymatous nephritis, the amount is often much larger. In this particular, however, it is very difficult to generalize, since the classification of the forms of nephritis on clinical grounds is none too sound at best.

Accidental albuminuria may be seen whenever there is opportunity for the admixture of blood or of inflammatory exudates with the urine in the urinary tract below the kidney proper. It may be due to menstrual blood, or blood from hemorrhage from trauma or new growth, to pus from pyelitis, ureteritis, cystitis, urethritis, vaginitis, or to addition of lymph, spermatic or prostatic fluid. Before drawing conclusions as to the existence of nephritis, due care must be taken to exclude any of these conditions as possible sources of error.

Qualitative Tests for Sugar.—Benedict's Test.—This is much to be preferred, not only on account of its delicacy but because of the permanency of the reagent.

The formula for Benedict's solution is:

Copper sulphate	17.3 gm.
Sodium citrate	173.0 "
Sodium carbonate (anhydrous)	100.0 "
Distilled water, to make 1000 cc.	

The sodium citrate and the sodium carbonate are dissolved by heating with 600 cc. of water, after which the resulting solution is filtered through a folded filtrate into a graduate and the total volume is made up to 850 cc. with water. The copper sulphate is dissolved separately in 100 cc. of water, when the bulk of this is made up to 150 cc. The sodium carbonate and citrate solution is now poured into a large beaker and the copper sulphate is added slowly with continual stirring.

Procedure.—In a test-tube place 5 cc. of the reagent and add not more than 8 to 10 drops of the urine. Boil the mixture thoroughly and allow to cool spontaneously. If glucose be present, the entire body of the solution will show a precipitate, ranging from green to red in color, according to the sugar content of the urine. In the absence of sugar, the solution remains quite clear or shows only a faint bluish turbidity.

Fehling's Test.—The formulæ for the two Fehling's solutions are:

Solution No. 1. Copper Sulphate Solution.

Copper sulphate	34.65 gm.
Distilled water, to make	500.00 cc.

Solution No. 2. Alkaline Tartrate Solution.

Potassium hydroxide	125.00 gm.
Rochelle salts	173.00 "
Water, to make	500.00 cc.

The solutions are kept in separate bottles, and small portions as needed are prepared by mixing equal quantities.

Procedure.—Fehling's solution should be prepared fresh daily, by mixing equal quantities of the two solutions. After the mixture has been made, boil a little in a test-tube to make sure that no reduction occurs.

To about 5 cc. of hot Fehling's solution add a few drops of urine, boil, and continue adding urine, a few drops at a time, until there are equal quantities of urine and Fehling's solution. Sugar is indicated by yellow or red precipitate. The appearance of a greenish color means nothing. In case of doubt, allow the tube to stand. When sugar is present, a distinct precipitate settles to the bottom of the tube.

Fermentation Test.—A doubtful result with either of the foregoing reduction tests should be confirmed by a fermentation test, since substances other than glucose, such as uric acid, formalin, creatinine, chloroform and simple aldehydes may give a slight reaction.

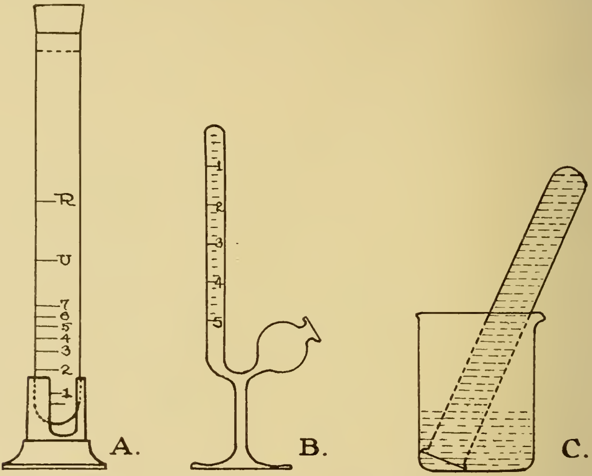


Fig. 63.—*A*, Esbach albuminometer; *B*, Einhorn saccharometer; *C*, improvised fermentation tube.

Two fermentation tubes (Fig. 63, *B*) are employed. In one the urine to be examined is placed after it has been thoroughly mixed with about one-sixteenth of a fresh cake of compressed yeast. The second tube is filled with normal urine, similarly mixed with an equal amount of yeast. The two tubes are kept at room temperature or may be placed in the incubator. The formation of gas in the upright denotes the presence of glucose providing the normal urine shows no gas formation.

In the absence of fermentation tubes, ordinary test-tubes may be employed, filling the tube with the urine and yeast mixture, and then setting it upright, with the opening down immersed in a beaker of the same urine. (Fig. 63, *C*).

Phenylhydrazine Reaction.—This reaction may be used to control the results of the reduction tests and has the advantage over the fermentation test that the results may be obtained in much shorter time. The reaction depends upon the formation of an osazone, which may be identified by its characteristic crystals. The test is an excellent one, since there are practically no substances except the carbohydrates which gave the reaction. Levulose and dextrose give identical osazones. The osazone formed by maltose can be identified by its crystals, and lactose will not give the crystals when the test is carried out as directed.

Kowarsky's method is preferred for clinical work. Five drops of the phenylhydrazine (the base) are mixed in a test-tube with 10 drops of acetic acid and 1 cc. of a saturated solution of sodium chloride. A curdy mass results, when 2 or 3 cc. of the urine to be examined and 4 or 5 cc. of water are added. The mixture is heated for about two minutes over the flame. Care should be taken to avoid bumping. This may be accomplished by constant shaking or by putting some short bits of glass tubing in the test-tube. When glass tubing is used, the fluid should be poured into another test-tube to cool. Frequently the crystals appear rapidly. If they do not, at least thirty minutes should be given, since this time may be required for crystallization when the amount of sugar is small. The deposit should be removed with a pipette, placed upon an object slide, and examined under the low power of the microscope. The crystals are yellow and needle-shaped, grouped in clusters or sheaves.

Significance of Glycosuria.—Normally, the urine contains very minute quantities of sugar, but not enough to be detected by the ordinary qualitative tests. On this account, Benedict has suggested using the term glycuresis instead of glycosuria. The normal glucose content of the circulating blood is about 0.1 per cent. When the concentration rises to 0.2 per cent. or thereabouts, constituting a hyperglycemia, glucose is secreted by the kidney into the urine. The occurrence of hyperglycemia and the resultant glycosuria may be due to defective storage of the sugar derived from the food and from endogenous sources, to defective oxidation of sugar, to abnormal permeability of the renal filter (phloridzin diabetes), or to an excessive ingestion of glucose, exceeding temporarily the capacity of oxidation and storage (alimentary glycosuria).

Glycosuria may be produced by the administration of the extracts of certain glands, notably the adrenal and the thryoid, and by the use of drugs, such as phosphorous, arsenic and ether. It may be seen in brain injuries, after severe burns and in cirrhosis of the liver.

It is evident from the enumeration of the conditions under which glycosuria occurs, that it may be transient or permanent. When sugar is found in the urine, it is well to repeat the test on another day and to be certain that the manifestation is not a transitory or accidental matter. The need for caution in this matter is well recognized by insurance companies.

Clinically the glycosuria of greatest interest is that associated with diabetes mellitus, where sugar accumulates in the blood to be excreted by the kidneys because of deficient oxidation and storage in the body, due in many cases to pancreatic disease. In contradistinction to transitory glycosuria, the glycosuria of diabetes is accompanied by definite symptoms. In diabetes the quantity of urine excreted is large, occasionally reaching 20 liters, and the amount of sugar may vary from a trace to as much as 10 per cent. The urine is usually pale, acid, and of high specific gravity. Acetone and diacetic acid may be present in varying amounts and albumin is commonly seen.

Detection of Other Sugars.—From a clinical standpoint, glucose is the only sugar found in the urine sufficiently frequently to make it of especial significance. *Lactose* is occasionally seen in the urine of nursing mothers especially when there is an obstruction to the flow of milk, or when nursing has been stopped. Lactose reduces Fehling's or Benedict's solutions, but does not give the fermentation test. Therefore when a reducing substance is found in the urine of nursing mothers, lactose should be thought of and should be carefully excluded before making a diagnosis of diabetes mellitus. In carrying out this test, it is important to use a pure culture of yeast to prevent bacteria ordinarily present in the commercial compressed variety from breaking down the lactose into a fermentable sugar. If a pure culture be not available, the fermentation test may be performed with the ordinary compressed yeast provided that the test be interrupted at the end of a few hours, when glucose should give some evidence of gas formation while lactose should not. In the amount in which it is present in the urine, it is not at all likely to give the phenylhydrazin test.

The determination of *maltose* has no practical clinical application. It may be demonstrated by determining the nitrogen content of the osazone formed, or by the discrepancy between the quantitative estimation as performed by the polariscope and by the reduction tests, the dextrorotary power being much greater than that of glucose.

MICROSCOPIC EXAMINATION.

The microscopic examination of the urine is of great importance and should not be omitted when albumin is absent. It should form part of the routine examination, and time spent in this direction will benefit the practitioner much more than that spent in most quantitative determinations. It is well recognized that a characteristic of the urine in chronic interstitial nephritis is the absence of albumin in a urine of low specific gravity with the presence of hyaline casts. Then too, the presence of pus cells, which may not be present in sufficient quantity to give the albumin tests, may give an invaluable clue to infection in the urinary tract.

The student should take pains to examine as many normal specimens as possible, so that he may become thoroughly familiar with normal findings, and may come to know from experience the number of leukocytes and epithelial cells which are to be expected in male and female urine. The latter is of particular importance, since the normal findings are quite different in the two sexes when voided specimens are examined, though catheterized specimens are practically the same under normal conditions.

The urine should be centrifugalized in a centrifuge tube with a conical tip (Fig. 61, *d*). If a centrifuge be not accessible, it may be allowed to stand for several hours, in a vessel with a conical bottom (Fig. 61, *c*).

In order to remove sediments press the pipette firmly at one end with dry forefinger and lower the other end to the bottom of the containing vessel. Rotating the pipette between thumb and forefinger will allow a little of the sediment to enter the pipette. Withdraw the pipette and allow the contents to escape on to a glass slide. This is now ready for examination under the microscope.

Darken the field by lowering the Abbé condenser and by almost completely closing the iris diaphragm just beneath the stage. First, examine the slide without a cover-slip, using the low power, to hunt for casts and crystals. Then put on a cover-slip and with high power examine for leukocytes, red blood-cells, epithelium and bacteria. A well-darkened field is absolutely essential if hyaline casts are to be found.

The constituents to be examined for may be divided as follows:

1. Organized elements, including casts, cylindroids, epithelial cells, leukocytes or "pus-cells," red blood cells or shadows of red cells, spermatozoa, mucous cylindroids, bacteria, yeasts, animal parasites.

2. Unorganized elements, including uric acid crystals, sodium urate (amorphous and crystalline), ammonium urate, hippuric acid, ammonium magnesium phosphate (triple phosphate) crystals, amorphous phosphates, calcium phosphate crystals, calcium oxalate crystals, calcium carbonate crystals, cystine, leucine, and tyrosine.

Organized Elements.—**Casts.**—Casts are formed in the urinary tubules, and in reality constitute an impression or casting of the tubule, which serves as a mould (Plate V). The substance of a cast is probably colloidal, whose exact source, whether a transudate from the blood or material from degenerated renal cells, is not settled. Casts, especially hyaline casts, do not possess great refractility and will be readily overlooked unless the field be well darkened. No significance should be attached to the absence of casts in alkaline urine, since they dissolve readily in an alkaline medium. The cast is a pale cylinder with slightly rounded ends. The relation between diameter and length is quite variable. Various types of casts are noted, the classification being based on morphology. When the cast is filled with granules it is referred to as a finely or coarsely *granular cast* according to the size of the granules.

There are also *blood casts, pus* (or leukocyte) *casts, epithelial casts, fatty casts,* depending upon the morphological element which has been incorporated in the hyaline matrix of the cast itself. These are of significance since they indicate that the particular morphological element has been incorporated in the kidney tubule itself and, therefore, has been "shed," as it were, in this place and not lower in the urinary tract. *Waxy casts* are light yellow, have sharply defined outline, and are much more refractile. They show a tendency to transverse splitting. *Fibrin casts* are highly refractile and yellowish brown in color, occasionally showing ragged fractures. Cells may be incorporated within their substance. They are not actually composed of fibrin. They are seen in acute renal conditions and should not be confused with waxy casts, which are of much more serious import. *Fatty casts* contain epithelium, which has undergone fatty degeneration. Often degeneration is so far advanced that cellular details cannot be made out. The droplets stain red with Sudan III or black with osmic acid.

Mucous Cylindroids.—These are long tapering bodies which are thinner than either casts or cylindroids.

Significance of Casts in the Urine.—Hyaline casts are found in various forms of nephritis. Their presence is not to be interpreted as absolute evidence of the existence of nephritis, since they may be found where there is only a temporary irritation of the kidneys (as after unusual exercise, during severe infection, or ether anesthesia); indeed, whenever the kidney undergoes marked circulatory change or toxic disturbance.

Granular casts, however, usually have more serious import, especially if found repeatedly, and may be interpreted as indicative of nephritis. It has been claimed that the coarser the granules, the more severe is the nephritis.

Fatty and waxy casts are both found in extremely advanced forms of nephritis. Waxy casts are not necessarily a sign of waxy kidneys, indeed, the amyloid nature of this form of casts cannot be demonstrated in all cases. The occurrence of this form of cast is a sign of grave prognostic import. *Blood, epithelial, and pus casts* point to the fact that the incorporated cells have been shed in the kidney itself and their presence must be interpreted accordingly. Epithelial casts indicate a destruction of renal epithelium and are seen in advanced nephritis. *Pus and leukocyte* casts point to an infectious process in the kidney substance, frequently a pyelonephritis.

Cylindroids.—Cylindroids resemble casts in their chemical nature and general structure except that they taper at one or both ends. It is possible for this tail-like end to be broken off so that the remaining portion of the cylindroid will present the exact appearance of hyaline cast. Differentiation cannot be made in such cases, except indirectly, by careful search of the sediment and finding other similar elements. The significance of cylindroids is not settled, but it is rather gencrally accepted that their presence does not point to renal damage (Plate V).

Mucus Threads.—These are classed by many writers with the cylindroids. They are even less refractile, and are long, thin, tapering threads of ribbon-like rather than cylindrical structure. Chemically, they differ from cylindroids, being of mucoid character. They compose the nubecula. No diagnostic significance is attached to them.

Leukocytes.—Leukocytes should be readily recognized, particularly when the high power of the microscope is employed, bearing in mind the presence of a nucleus and the granular appearance of the cytoplasm. The leukocytes are well preserved in acid urine, but tend to dissolve in alkaline urine. The addition of a drop of dilute (5 per cent.) acetic acid accentuates the nuclei of the leukocytes, making them appear darker, while it hemolyzes the red blood cells. Occasional leukocytes are seen in normal urine, the number being somewhat greater in specimens from women.

An increased number of leukocytes (*pyuria*) occurs in infection of the genito-urinary tract. There is nothing about the number or appearance which may be taken as characteristic either of the type of infection or the locality involved.

Red Blood Cells.—Generally speaking, red blood cells are never seen in normal urine. When red cells are present in the urine, the condition is spoken of as hematuria, and is to be distinguished from hemoglobinuria, in which only free hemoglobin is present.

Red blood cells may be distinguished from white cells with the higher power of the microscope, and with the aid of acetic acid, as has been mentioned previously. The corpuscles may appear as mere "shadows," due to laking of the corpuscles.

Red blood cells are seen in large numbers, of course, at the time of the menstrual period. Pathological conditions which may lead to their appearance are the presence of calculi, injuries to the genito-urinary tract or the kidneys, inflammatory processes, notably tuberculosis, malignant disease of the kidneys or genito-urinary tract, of which papilloma of the bladder serves as an example, acute infectious nephritis, acute toxic nephritis, notably that due to mercury, where erythrocytes may be seen, even after the therapeutic use of the drug. Red cells may be seen in the chronic forms of nephritis, especially with acute exacerbations, in chronic congestions of the kidney, such as that occurring during decompensation of chronic heart disease, in the various form of purpura, occasionally in hemophilia when the hemorrhage is in the genito-urinary tract, and in infection with filaria or distoma hamatobium. There is also a so-called "functional" or idiopathic hematuria.

Epithelial Cells.—These are normally seen in small numbers. In female urines which have been voided, the normal number is large. These are pavement epithelial cells from the introitus. Epithelial cells (Plate V) should be divided into three classes:

(*a*) Large flat cells, the form most frequently seen, may come from ureters, bladder, or vagina.

(*b*) Round cells, rather larger than leukocytes with large, clearly defined nuclei. These come from the uriniferous tubules and from deeper layers of the mucous membrane of other portions of the urinary tract.

(*c*) Caudate cells, with distinctly tail-like prolongation of the cell body, usually coming from the renal pelvis or the neck of the bladder.

It should be remembered that the cell forms are not definitely characteristic of any one locality in the genito-urinary tract. To attempt to localize the exact site of inflammatory process in the genito-urinary tract by the identification of a certain type of epithelium is a mistake which is not made nowadays by well-informed clinicians. The identification of a predominating type of epithelium when taken in conjunction with other findings may be of some assistance.

Yeast Cells.—These are occasionally seen, especially in urines which have been kept some time in poorly cleaned receptacles. They are not infrequently confused with red blood cells by the inexperienced. They should not cause confusion, since the yeast cells are irregular in size and frequently show budding.

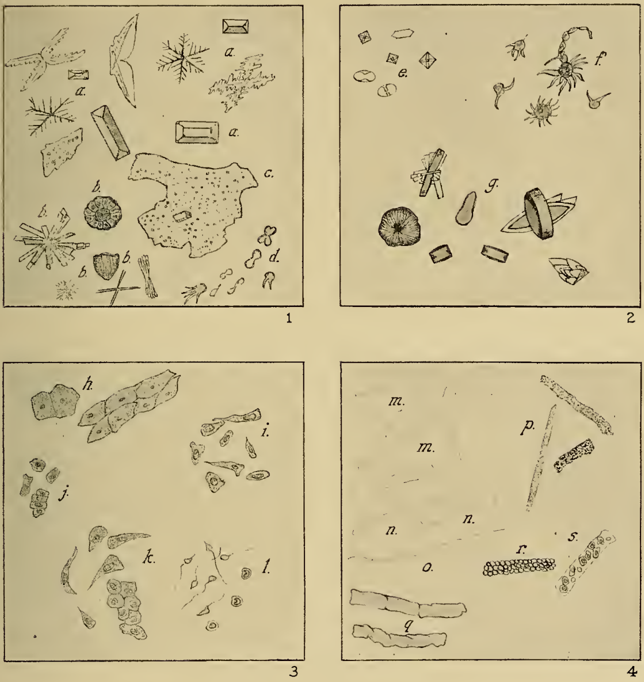
Bacteria.—Before drawing conclusions from urinary bacteria, one should satisfy himself that the specimen had been voided recently into a clean container, and that the organisms are not due to contamination.

Unorganized Elements.—There is no doubt that the subject of urinary crystals and amorphous deposits has received vastly more attention in the past than has been justified by the clinical knowledge derived from the study. Certainly the clinical significance of the various forms of crystals is not great. Their identification, however, completes the description. Triple and amorphous phosphates are the rule in alkaline urines and their presence always suggests the possibility of decomposition. Calcium oxalate crystals are observed after the ingestion of tomatoes, asparagus, rhubarb, oranges, etc., and are also seen in diabetes mellitus, etc. It is stated that calcium oxalate and uric acid crystals are found in cases with a tendency toward the formation of urinary calculi. Uric acid crystals are seen in a highly concentrated, acid urine. Leucine and tyrosine are found in acute yellow atrophy of the liver, in acute phosphorus poisoning, in cirrhosis of the liver, in cases of severe typhoid.

Amorphous Deposits.—The gross appearance of the amorphous deposits has been described previously. A pink deposit is usually composed of urates. It dissolves when the slide is heated gently. Urates may be white also. Amorphous phosphates are white in color and dissolve readily with the addition of dilute acetic acid. The microscopical appearance of an amorphous deposit is the same whether it is due to urates or phosphates, the slide looking as though it were covered with fine, moderately refractile granules.

Sediments in Acid Urines (Plate V).—The amorphous sediments consist of urates of potassium and sodium. Their appearance has

PLATE V



Urinary Sediments.

1. Deposit in alkaline or neutral urine: *a*, ammonio-magnesium phosphate; *b*, "neutral" calcium phosphate; *c*, plates of "neutral" calcium phosphate; *d*, acid ammonium urate.

2. Deposit in acid urine: *e*, calcium oxalate ("dumb-bell" and "envelope" forms); *f*, acid ammonium urate; *g*, uric acid.

3. *h*, vaginal epithelium; *i*, epithelium from ureter; *j*, renal epithelium; *k*, epithelium from renal pelvis; *l*, spermatozoa.

4. *m*, hyaline casts; *n*, cylindroids; *o*, mucus threads; *p*, granular casts; *q*, waxy casts; *r*, fatty cast; *s*, leukocyte and red blood cell cast.

been described. They dissolve readily with heat or upon the addition of acetic acid.

Uric Acid.—When uric acid separates from the urine, the crystals are colored brown, though pure uric acid gives colorless crystals. It crystallizes into a number of forms, wedges, whetstones, rhombic prisms, irregular rectangular or hexagonal plates, dumb-bells, etc. Uric acid crystallizes from an acid urine, particularly when cold.

Calcium Oxalate.—Calcium oxalate appears as small dumb-bells, or more frequently as a refractile, small octahedra with a cross connecting the corners. In appearance, the octahedra resemble the back of square envelopes. The crystals are precipitated in acid urine, but may be seen in decomposed alkaline urine because of their insolubility in alkalies.

Cystine.—Cystine is only rarely found in the urine. It is found in acid urines in the form of highly refractile, colorless, six-sided plates.

Leucine and Tyrosine.—Leucine and tyrosine always occur together in the urine, and are found in acid urine in acute yellow atrophy of the liver, acute phosphorus poisoning, etc. Leucine crystallizes as small, highly refractile, yellowish, spheroids which show both radial and concentric striation, while tyrosine forms bundles of fine needles.

Bilirubin or Hematoidin.—Both substances appear in the form of yellow-red needles or rhomboids, soluble in chloroform and potassium hydrate.

Hippuric Acid.—Crystals of hippuric acid are found in the urine rather infrequently and their presence is due to the nature of the diet, since they are especially apt to be seen after the use of certain fruits or the administration of benzoic acid. They appear as colorless needles or prisms, pigmented as are the crystals of uric acid as they are found in the urine. They are soluble in alcohol and ether.

Sediments in Neutral or Amphoteric Urine.—In addition to those which have been enumerated above as occurring in acid urine, neutral calcium phosphate may be found, and rarely, ammonium magnesium phosphate.

Neutral Calcium Phosphate.—Neutral calcium phosphate may appear in the form of slender pyramids united at the apices to form rosettes. They are soluble in acetic acid and 20 per cent. ammonium carbonate. Neutral calcium phosphate also is seen as a scum floating on the top of the urine or adhering to the outside of the pipette. Microscopically, it appears as large plates or sheets, with very irregular edges.

Sediments in Alkaline Urine (Plate V).—The amorphous phosphates have been described previously. In addition, we may have crystals of calcium carbonate, ammonium urate, and of ammonium-magnesium phosphate.

Ammonium-magnesium Phosphate (*Triple Phosphates*).—Ammonium-magnesium phosphate crystallizes in the form of long prisms (the so-called "coffin-lids"), or in long feathery crystals. The latter are usually grouped together, four being united at their bases to form a letter H. They are readily soluble in acetic acid.

Calcium Carbonate.—Calcium carbonate may form amorphous masses or may crystallize as spheres or dumb-bells. They dissolve readily upon the addition of acetic acid with the evolution of carbon dioxide gas.

Ammonium Urate.—Ammonium urate is ordinarily present in the form of dark yellow or brown spheres, often covered with spines. They are readily soluble in hydrochloric acid.

Identification of the Inorganic Sediments.—Chemical means may be of assistance in the identification of the sediments. The following table given by Sahli is useful in identifying the principal varieties.

I. Readily soluble upon heating: Urates.
II. Insoluble or soluble only with difficulty upon heating.
    A. Soluble in acetic acid.
        1. Phosphates (no effervescence).
        2. Calcium carbonate (with evolution of gas).
        3. Ammonium urate (with microscopic precipitate of uric acid).
    B. Insoluble in acetic acid.
        1. Soluble in hydrochloric acid; the last three also soluble in ammonia.
            (*a*) Calcium oxalate.
            (*b*) Leucin, tyrosine, xanthine, cystine.
        2. Insoluble in hydrochloric acid.
            (*a*) Uric acid.
            (*b*) Calcium sulphate.

Another helpful classification is that of Wood, who divides the sediments according to the reaction of the urine in which they occur.

I. Sediments in acid urine:
    A. Amorphous urates (quadrates of sodium and potassium).
    B. Uric acid.
    C. Calcium oxalate.
    D. Cystine.
    E. Leucine.
    F. Tyrosine.
    G. Bilirubin or hematoidin.
    H. Hippuric acid.
II. Sediments in neutral or amphoteric urine.

In addition to those above, neutral calcium phosphate may be found and ammonium-magnesium phosphate rarely.

III. Sediments in alkaline urine.
    A. Amorphous phosphates (tricalcium and trimagnesium phosphates).
    B. Calcium carbonate.
    C. Ammonium urate (spheres or "thorn apples").
    D. Triple phosphates or ammonium-magnesium phosphate.

SPECIAL QUALITATIVE TESTS.

A number of qualitative tests may be performed on special occasions, either when the clinical findings or when the laboratory data obtained on the routine examination point to their desirability.

Tests for Bile Pigments.—When the urine is highly colored, when the foam shows yellow color, when jaundice is present, or when clinical indications suggest an obstruction of the biliary tract or inflammatory conditions of the gall-bladder or biliary passages, tests for bile should be made.

Foam Test.—Shake the urine in a bottle. A brownish-yellow foam indicates the presence of bile. This test should not be accepted as final, but should be confirmed by one of those given below.

Gmelin's Test.—Under the 5 cc. of urine in a test-tube, run from a pipette 5 cc. of concentrated nitric acid, carefully and slowly, as in Heller's test for albumin. If bile be present, there will appear concentric rings of various colors.

Trousseau's Test.—With a pipette run 5 cc. urine under 5 cc. of very dilute tincture of iodine (1 part of tincture of iodine plus 39 parts of 95 per cent alcohol). Bile pigments will give a dark-green ring at the junction of the liquids.

Significance of Bile Pigments.—Bile pigments may gain access to the general circulation and be excreted by the urine whenever there is an obstruction of the free outflow of bile from the liver into the intestinal tract. This obstruction may be in the common duct, in the smaller biliary passages, or in any of the intermediary passages. Mechanical causes are stones in the biliary passages, pressure from without by new growths such as a tumor of the pancreas, a tuberculous lymph gland, or adhesions. Jaundice is a conspicuous sign in the acute catarrhal jaundice accompanied by gastro-intestinal disturbances, and is seen in cirrhosis of the liver, acute yellow atrophy, anemia of hemolytic jaundice, and in cholangitis.

Determination of Bile Acids.—The determination of bile acids requires the presence of at least 0.5 per cent. This impairs the value of the procedure, since this amount is often not present. The detection of bile pigments is therefore much the method of choice on account of the possibility of detecting readily much smaller traces. The detection of both pigments and acids is a needless refinement since pigments and acids are usually present under the same conditions.

There is no test for bile acids which is both simple and reliable. Hay's tests depended upon an alteration in the surface tension. Flour of sulphur is dusted over the surface of the urine, which is placed in a beaker or open vessel. It was assumed that the sulphur would sink because of the reduction in surface tension when bile acids are present and would float on the surface when absent.

Tests for Acetone and Diacetic Acid.—Examination should be made for these bodies whenever sugar is found on the routine examination, when the clinical history indicates diabetes mellitus, or when the patient is in coma.

Tests for Acetone.—In performing any of the tests for acetone except Rothera's, the urine should be distilled, using either a retort or a flask fitted with a cork through which is passed a long piece of glass tubing (Fig. 64). The urine is acidulated with 5 per cent. acetic acid and distillation is allowed to proceed until about one-third of the original volume has been collected. To the distillate add 5 drops of 10 per cent. HCl and redistill.

Rothera's Test.—Preference is given to this method. To 5 or 10 cc. of urine add about 1 gm. of ammonium sulphate and 2 or 3 drops of a freshly prepared 5 per cent. solution of sodium nitroprusside. Stratify strong ammonium hydroxide upon the mixture. When acetone is present, a characteristic permanganate color develops slowly. The delicacy of the test is 1 to 20,000.

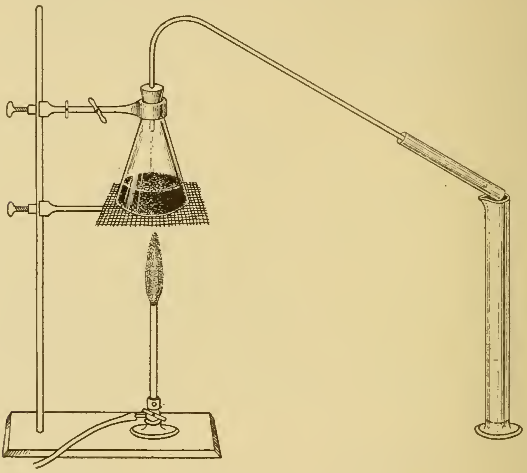


Fig. 64.—Distillation of urine for acetone tests.

Gunning's Iodoform Test.—To 5 cc. of distillate add a few drops of Lugol's solution and enough ammonia to form a black precipitate. When the black precipitate appears, add a few more drops. Allow the tube to stand until a yellowish sediment appears, and examine this microscopically for iodoform crystals. These are six-sided plates and six-pointed stars.

Legal's Test.—To 5 cc. of distillate, add a few drops of a freshly prepared concentrated aqueous solution of sodium nitroprusside. Render the mixture alkaline with 10 per cent. potassium hydroxide.

Normally a red color appears. This is due to creatinine. Add an excess of glacial acetic acid and if acetone be present, the color will be intensified to almost a purple. If acetone be absent, a yellow color is seen.

Diacetic Acid.—*Gerhardt's Test.*—Put 5 cc. of urine in a test-tube and add 5 per cent. aqueous solution of ferric chloride, drop by drop, as long as precipitation occurs. If a heavy precipitate of phosphates occur, filter and to the fitrate add a few drops of the ferric chloride solution. A Bordeaux red color is produced by diacetic acid.

The reaction is a general one for ketone bodies, since a reaction is given by acetone as well as diacetic acid.

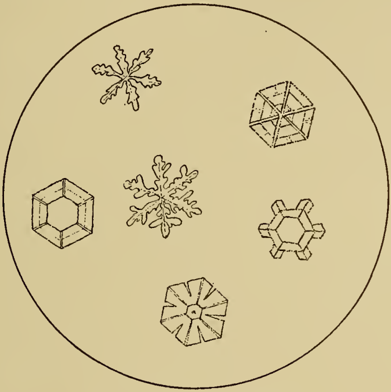


Fig. 65.—Iodoform crystals.

Test for β-Oxybutyric Acid.—*Hart's Test.*—Twenty cc. of urine are placed in an evaporating dish and an equal quantity of water is added. The mixture is boiled down to about 10 cc. to free it of the acetone and diacetic acid. Water is added to bring the volume to 20 cc. and the mixture is divided equally between two tubes. One cc. of hydrogen peroxide is added to one of the tubes and to each tube is added 0.5 cc. of glacial acetic acid, with a small crystal of sodium nitroprusside. After mixing, 2 cc. of concentrated ammonia is layered over the mixture in each tube and the tubes are allowed to stand. If β-oxybutyric acid be present, the contact ring should show a purplish red color in the tube to which hydrogen peroxide had been added, while it should be colorless in the control tube to which no peroxide has been added.

Significance of the Acetone Bodies.—The foregoing substances, beta-hydro-oxybutyric acid, diacetic acid and acetone, are closely related chemically and the clinical significance of their presence in the urine will be considered at the same time. Beta-hydro-oxybutyric acid is the mother-substance, oxidation yields diacetic acid, and oxidation of this in turn gives acetone, though diacetic acid is considered the mother-substance by some. The chief source of the bodies is from the fats, which are improperly metabolized. Fat is less readily oxidized than carbohydrates and requires "the fire of burning sugar to consume it. If the carbohydrate fires do not burn briskly enough, the fat is incompletely consumed; it smokes, as it were, and the smoke is represented in metabolism by the ketones and derived acids." (MacLeod.) Lack of combustion of the carbohydrates may be brought about by deficient intake, as in starvation, or by faulty combusion, as in diabetes. The accumulation of these acetone substances in the body leads to a type of acidosis which is seen in diabetes, cholera, in starvation and in other conditions. Obese individuals not infrequently show acetone bodies in the urine during preoperative and postoperative starvation.

In milder cases, only small amounts of acetone may be excreted, but in more severe cases diacetic acid and β-hydro-oxybutyric acid also are found in their urine. The amount of these substances present serves as a guide to the degree of acidosis. The exact quantitative determination is time-consuming, and the amount may be arrived at indirectly by the determination of the ammonia, since the diacetic acid and β-oxybutyric acid in the organism combine with ammonia and are excreted as ammonia compounds. Magnus Levy states that the normal daily excretion of ammonia is 0.7 gm. and that if the amount is raised to 2 gm. it is equivalent to about 6 gm. of oxybutyric acid, while 5 gm. is equivalent to 20 gm. of oxybutyric acid.

The continued finding in the urine of all three acetone bodies with an increased amount of ammonia and the persistence of these bodies in spite of withholding carbohydrates, is rather generally accepted as basis for a poor prognosis. These findings may have little relation to the quantity of sugar excreted. In mild cases, the amount of sugar may be small, and may disappear upon limitation of the carbohydrate intake. With this limitation of carbohydrates, the amount of acetone may increase but diminishes as the organism becomes accustomed to burning fat.

Indican.—*Obermayer's Test.*—In a test-tube place about 10 cc. of clear urine and 10 cc. Obermayer's reagent (made by adding 2 gm. of ferric chloride to 1000 cc. hydrochloric acid) and add 5 cc. of chloroform. Shake well and cork the tube. If indican be present, the chloroform will take the color of indigo blue. Normal urine shows only a faint blue color. The depth of the coloration serves as an approximate measure of the amount of indican present. If desired, the urine may be mixed with the hydrochloric acid and two or three drops of 5 per cent. ferric chloride may be added instead of using Obermayer's solution. Indican is derived largely from the putrefaction of protein which occurs in the intestinal tract, protein cleavage resulting in the formation of indol and skatol. Indol is absorbed in the circulation to indoxyl, which is conjugated in the liver with sulphuric acid to form indoxyl-potassium-sulphate, or indican. Increased quantities are found in the urine in conditions which permit intestinal putrefaction, such as intestinal obstruction, ileus, and peritonitis. It is only occasionally found in cases of simple constipation. When the large bowel is occluded, indican does not appear unless there be also stasis of the ileum. Decomposition of protein in other parts of the body may give rise to an increase in the amount of urinary indican, such as gangrene of the lung, empyema, etc.

While indican may be determined quantitatively by Ellinger's method, the technic is not such as would recommend it for clinical work. The clinical value of the indican test, performed qualitatively, is rather limited and is seen chiefly in connection with mild gastro-intestinal disturbances of the functional type, marked by symptoms of headache and lassitude.

Blood.—Blood may occur in the urine in the unchanged form, when the corpuscles may be identified microscopically, the condition being referred to as hematuria; or it may be excreted as a solution of hemoglobin or one of its derivatives, this condition being referred to as hemoglobinuria. The detection of red blood corpuscles has been discussed previously. Hemoglobin is easily identified by the spectroscopic method. Since hemoglobin is a protein, urine containing it would give the usual tests for protein bodies.

Benzidine Reaction.—About 2 cc. of urine are placed in a test-tube and are acidified with glacial acetic acid. If the blood be unchanged, this serves to lake the corpuscles and to convert the hemoglobin to acid hematin. About 5 cc. of an alcoholic solution of benzidine are added (prepared by making a saturated solution in 95 per cent. alcohol heated over a water-bath). The contents of the tube are thoroughly mixed and 5 cc. of hydrogen peroxide solution are added. A Prussian blue color indicates the presence of blood pigment. Care should be taken that the solution of hydrogen peroxide is a potent one.

The test has been simplified by the use of compressed tablets containing benzidine and sodium perborate according to the method of Roberts. A tablet is soaked with the urine or with a watery emulsion of the feces to be examined, and upon it is then placed a drop of glacial acetic acid. The oxygen is furnished by the sodium perborate instead of by hydrogen peroxide. This modification has the advantage of ready availability and simplicity, but is not quite as delicate as the method previously described.[1]

Spectroscopic Test.—A small pocket spectroscope is required (Fig. 66). The urine should be filtered and placed in a test-tube and a layer of distilled water run over it with care to avoid shaking, so that gradual mixing may give a column of fluid with varying dilution of hemoglobin from the bottom to the top of the tube. The tube is placed in position in the holder of the spectroscope and the absorption bands are located, using direct daylight. The bands may be those of oxyhemoglobin, of reduced hemoglobin, of methemoglobin, or rarely, of hematin.

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[1] These tablets are prepared by E. R. Squibb and Sons.

Oxyhemoglobin shows two bands between *D* and *E; reduced hemoglobin,* one band between *D* and *E; methemoglobin,* if the solution be acid, by an additional dark band between *C* and *D* (Plate VI).

If the worker be not entirely familiar with the spectra in question, it would be advisable to prepare control tubes with known solutions for the sake of comparison. Oxyhemoglobin would be prepared by making a dilute solution of blood in distilled water. Reduced hemoglobin may be prepared by taking blood which has been diluted to show the two absorption bands of oxyhemoglobin and by adding to it a few drops of Stoke's reagent (a solution made with ferrous sulphate, 2 gm.; tartaric acid, 3 gm.; distilled water, q. s., to 100 cc. When needed for use, ammonium hydroxide is added to a small quantity drop by drop until the precipitate which forms at first entirely dissolves). Methemoglobin may be prepared by diluting a drop or two of blood with distilled water. To the dilution is added a few drops of freshly prepared 10 per cent. solution of potassium ferricyanide, and a few drops of acetic acid to acidulate.

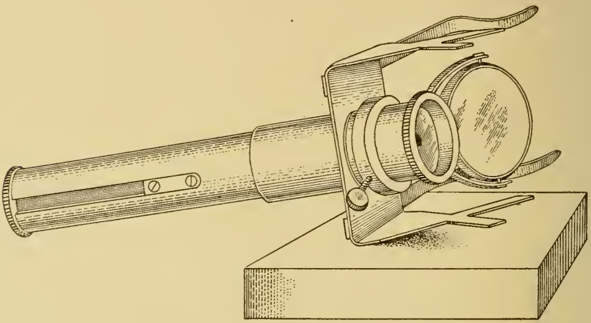
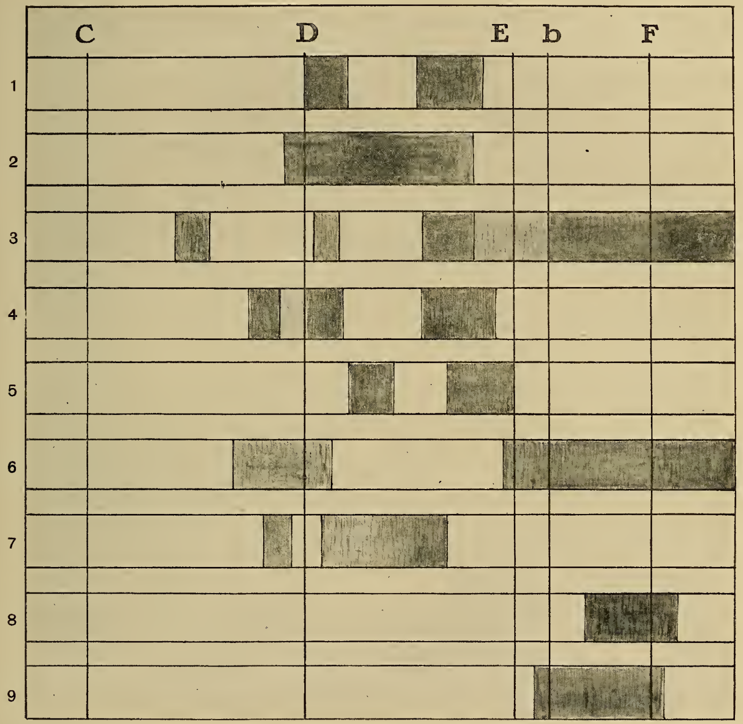


Fig. 66.—Spectroscope.

Significance of Hemoglobinuria.—The significance of hematuria has been discussed. Hemoglobinuria implies the presence of free hemoglobin in the circulating blood. This may occur after transfusions of blood, in severe form of malaria (so-called "black-water fever"), as a result of hemolytic poisons, such as snake venom, nitrobenzol, chloroform, pyrogallic acid, etc., and in paroxysmal hemoglobinuria.

Hematoporphyrin.—Hematoporphyrin is an iron-free derivative of hemoglobin normally found in minute traces. The amount is increased in sulphonal, trional, and tetronal poisoning. Spectroscopic examination is employed for its identification.

PLATE VI



Absorption Bands of Hemoglobin and Some of its Derivatives.

Absorption bands of (1) oxyhemoglobin; (2) reduced hemoglobin; (3) neutral methemoglobin; (4) alkaline methemoglobin; (5) carbon monoxide hemoglobin; (6) alkaline hematin; (7) acid hematoporphyrin; (8) urobilin or hydrobilirubin in acid solution; (9 urobilin or hydrobilirubin in alkaline solution after adding zinc chloride.

Procedure.—About 20 cc. of 10 per cent. sodium hydrate are added to 100 cc. of urine. The resulting mixture is filtered through filter paper, the filtrate discarded, and the precipitate washed with distilled water and partially dried by blotting it with filter paper. It is then scraped into a test-tube with a spatula and is dissolved by shaking with a mixture containing about 1 cc. of hydrochloric acid and 9 cc. of 95 per cent. alcohol. The solution should be filtered and examined with the direct vision spectroscope for the absorption bands of acid hematoporphyrin, one a narrow band slightly to the left of the *D* line and the other a much broader band between the *D* and *E* lines.

A control may be prepared by adding two drops of blood to 5 cc. of concentrated sulphuric acid.

Urobilin.—Ten cc. of urine are alkalinized with ammonia and 25 drops of 10 per cent. solution of zinc chloride are added. The mixture is allowed to stand a few moments when it is filtered to free it from phosphates. To the filtrate are added a few more drops of the zinc chloride solution. The tube should be held against a dark background and examined with a ray of strong light. An electric pocket flash light or the light from a head mirror may be utilized. When urobilin is present a distinct green fluorescence is seen.

The urine may be examined directly with the spectroscope. The absorption bands may be intensified somewhat by adding a few drops of tincture of iodine. In an acid solution the band is in the green portion, between the lines *b* and *F*. If desired, the zinc chloride test may be confirmed spectroscopically. The band will be broader and will be moved over somewhat to the left hand side of the spectrum, so that it will overlap the *b* line.

Urobilin or its precursor urobilinogen, exists in all urine. Its origin is not definitely settled, but the weight of opinion inclines to the following explanation. In the destruction of the erythrocytes, the liberated hemoglobin is carried to the liver, where the chromogen group yields the bile pigment, bilirubin. This is excreted as urobilinogen and urobilin. Both are reabsorbed into the blood and are excreted into the urine, which normally contains urobilin or urobilinogen. The excretion is greatly increased in certain conditions, in the hemolytic anemias, in the poisonings and in the infectious diseases characterized by destruction of blood, in cirrhosis of the liver, in acute yellow atrophy of the liver, in hemophilia, extra-uterine pregnancy, secondary syphilis, etc.

It is absent from the urine in cases of advanced nephritis where the impairment of the kidney prevents its excretion into the urine (Wood), and in cases where a complete obstruction of the common bile duct prevents the excretion of billirubin into the intestines and the consequent formation of urobilin (Barker).

From what has been said, it may be seen that a positive result with a qualitative test does not have any great clinical significance since urobilin or urobilinogen is found in the normal urine. A quantitative test might furnish information of considerable value serving particularly as an index of increased blood destruction, but because of its technic, it does not commend itself for clinical purposes. Barker feels that a negative qualitative test gives important confirmatory evidence of the presence of an icterus due to obstruction of the common duct.

Diazo Reaction.—Ehrlich's reagent should be freshly prepared by mixing one part of solution A with 50 parts of solution B. Equal quantities of this reagent and urine are placed in a test-tube and thoroughly shaken. The test is regarded as positive when the fluid assumes a deep cherry-red color, and the foam is distinctly pink. Normal urine results only in a brownish yellow color. A test should not be regarded as positive unless the foam be pink.

The formulæ for the two solutions are:

A

Sulphanilic acid	1 gm.
Hydrochloric acid (concentrated)	50 "
Water	1000 cc.

B

Sodium nitrite	1 gm.
Water	200 cc.

The nature of the substances which give this color reaction with the diazo bodies is unknown. The reaction is obtained in from 70 to 80 per cent. of the cases of typhoid in the first two weeks and in relapses. Its value is considerably impaired by the fact that positive reactions are also given in pneumonia, acute miliary and pulmonary tuberculosis, measles, scarlatina, sepsis, and trichinosis (Barker). Certain drugs also give the reaction, notably chrysarobin and naphthalin. Wood states that these reactions may be distinguished by the fact that with them the color is more permanent in alkaline solutions; that it does not fade appreciably upon the addition of strong mineral acids; that the foam is more yellow; and that a green precipitate does not appear upon standing.

The clinical value of the diazo reaction is extremely limited, for it appears in many diverse conditions. As a diagnostic test for typhoid fever, it is, in the writer's opinion, of very slight assistance, since it is given in a limited number of positive cases and is found in so many conditions which are most likely to give rise to confusion in making a differential diagnosis.

"Bence-Jones Protein."—Heat the urine gently, noting the temperature. If "Bence-Jones protein" be present, a turbidity may appear at as low a point as 40° C. It is convenient to stand a test-tube filled with urine in a beaker of water. A thermometer is held in the water, which is heated gradually to 100° C. When the temperature reaches 60° C., flocculent precipitation forms. Acidify the urine slightly with dilute acetic acid, and heat to 100° C. Most of the precipitate will disappear but will reappear upon cooling.

As a confirmatory test, it is well to precipitate the protein by the addition of 25 per cent. nitric acid. This precipitate should clear on heating and reappear on cooling. Further, the protein is completely precipitated by adding to the urine 2 volumes of 96 per cent. alcohol.

It is generally admitted that the Bence-Jones body is not an albumose, as it was at first regarded, but is a true protein, elaborated in the body under abnormal conditions, yielding on digestion primary and secondary albumoses, and characterized by a high aromatic radical content. It is found in many, though not all, cases of multiple myeloma, especially when the thoracic skeleton is involved (Simon), and occasionally in leukemia. Its occurrence has been reported in isolated cases of osteomalacia, carcinomatosis involving the osseous system, and one case of gunshot wound of leg.

Miller and Baetjer report that in a period of eighteen months they found seven cases of Bence-Jones proteinuria. One was a case of chronic myelogenous leukemia, one a case of true myeloma, and in the other cases there were no evidences of either of these diseases. Some of the latter were young and apparently healthy individuals showing hypertension and cylindruria despite renal function tests which gave apparently normal results. They advance the idea that these cases may be additional proof that Bence-Jones proteinuria may be an inborn error of metabolism.

They also suggest that possibly many instances of this form of proteinuria are missed in the urinary examinations as routinely performed simply because of the widely accepted idea of its rarity.

Nucleoprotein. — Place 10 cc. of urine in a beaker, add 3 volumes of water to prevent the precipitation of urates and acidify strongly acid with acetic acid. Turbidity indicates the presence of nucleoprotein. Positive identification is difficult. If albumin be present, it should be removed by boiling and filtering the urine before testing it.

Another method which has been employed is to perform the cold nitric acid test. Albumin or globulin will give a compact ring at the line of junction between urine and acid, while the precipitate of nucleoprotein is usually more diffuse, and will rise to a higher level, about 5 to 10 mm. above this line.

The chemistry of the nucleoprotein and nucleoalbumin bodies occurring in the urine is decidedly a moot question. A survey of the current literature and standard texts show that no two writers agree as to the nature of the substance which is precipitated by acetic acid in diluted urine in the cold, and that the identification of the bodies has not been completed by demonstration of the nuclein and phosphorus portions. Whatever may be the chemical structure and relationship of the bodies, it must be admitted that their clinical importance is not great, the important point being to avoid confusing them with serum albumin or globulin, whose presence in the urine is of so much greater import. This fact is recognized by certain of the insurance companies, which will accept applicants whose urine shows "nucleoprotein" as recognized by the foregoing tests while an albuminous urine would be ground for rejection.

QUANTITATIVE DETERMINATIONS.

Cautions.—It should be remembered that any quantitative determination has little value unless it is made with a twenty-four-hour specimen, in order that the total daily output of a given substance may be determined.

Albumin.—*Esbach's Method.*—An Esbach albuminometer tube is necessary (Fig. 63, *A*). Introduce urine to the mark *U*, and then carefully add Esbach's reagent (10 gm. of picric acid and 20 gm. of citric acid dissolved in 1 liter of water) to the mark *R*. Cork the tube, invert gently about 10 times so that mixing may be thorough, and place the tube in a test-tube rack, where it should stand for twenty-four hours. Then the upper level of the precipitate may be read on the engraved scale, which gives the number of grams of protein per liter of urine. If the precipitate comes to the mark 2, there are 2 gm. of protein per liter, or 0.2 per cent.

Tsuchiya's Method.—This method is more accurate and may be employed in the same way. The formula is: phosphotungstic acid, 1.5 gm.; concentrated hydrochloric acid, 5 cc.; alcohol (95 per cent.), 95 cc.

Purdy's Method.—Put 10 cc. of urine in a 15 cc. graduated centrifuge (Fig. 54, *g*) tube and add 3 cc. of 10 per cent. potassium ferrocyanide and 2 cc. of 50 per cent. acetic acid. Mix solutions thoroughly and allow the tube to stand for ten minutes. Centrifugalize for exactly three minutes at 1500 revolutions per minute in a centrifuge the radius of which is $6\frac{3}{4}$ inches to the tip of the tube. The amount of precipitate is noted and the result translated to grams of protein per liter of urine from the following table:

Volume of precipitate in graduated tube, cc.	Dry weight of protein to liter, gm.	Volume of precipitate in graduated tube, cc.	Dry weight of protein to liter, gm.
0.25	0.5	2.75	5.7
0.50	1.0	3.00	6.3
0.75	1.6	3.25	6.8
1.00	2.1	3.50	7.3
1.25	2.6	3.75	7.8
1.50	3.1	4.00	8.3
1.75	3.6	4.25	8.9
2.00	4.2	4.50	9.4
2.25	4.7	4.75	9.9
2.50	5.2	5.00	10.4

Sugar.—*Fermentation Method.*—This method is only approximately accurate, but has the advantage of simplicity. An Einhorn saccharometer is required (Fig. 63, *b*). About 15 cc. of urine are placed in a beaker and in it is ground up about one-sixteenth of a cake of compressed yeast, using a glass rod as a pestle. The resulting mixture is placed in a saccharometer tube. Care must be taken that there are no air-bubbles at the top of graduated tube, which should be completely filled with urine. The tube is placed in a warm room or in the incubator, and at the end of twelve hours the percentage of sugar is read at the upper level of the fluid in the tube. A control tube should be set up with normal urine mixed with the same amount of yeast.

If the specific gravity is high, pointing to the presence of a large quantity of sugar, the urine should be diluted by adding 1, 2, 3, or 4 times the amount of distilled water, to make a dilution whose specific gravity will be about 1.010. The final reading should be multiplied by 2, 3, 4, 5, as the case may be.

Lohnstein Saccharometer.—The instrument consists of a glass bulb connected with a U-tube, one arm of which is longer than the other and is fitted with two scales on which the percentage of sugar may be read, one adjusted for room temperature and the other for incubator temperature. The outfit should contain 12 cc. of mercury, which serves to separate the urine from the atmosphere. One-half cc. of urine is placed in the bulb over clean mercury and the same amount of thick emulsion of yeast is added. After the stopper has been greased with a mixture of equal parts of vaseline and yellow wax, it is inserted so that the apertures in the stopper and the neck of the bulb correspond, allowing communication between the space inside the bulb and the outside air. The apparatus is then tipped so that the level of the mercury in the long arm coincides with the zero of the scale, when the stopper is turned enough to close the opening. A weight is placed on the stopper and the apparatus put in the incubator for six hours, when the result may be read at incubator temperature on the scale adjusted to 35° C. When an incubator is not available, fermentation may be allowed to proceed about twenty-four hours at room temperature. The chief disadvantage is the difficulty in cleaning.

Reduction Methods.—The reduction methods are generally employed. A number have been devised, all of which are modifications of Fehling's test. Purdy and Pavy's methods have certain points of advantage, chief of which is the exceedingly sharp end-point. Purdy's solution keeps for a period of months, much better than any of the copper solutions proposed prior to Benedict's. The principal objections to it are the liberation of ammonia fumes, which are annoying in a laboratory, and the lack of permanence as compared with Benedict's solution. The method of Benedict is almost ideal and has come into general use in this country. If the estimation be carried out according to Myers' suggestion with the use of a small quantity of the reagent, the performance of a determination becomes a matter of extreme simplicity.

Fehling's Quantitative Method.—Equal quantities of Fehling's copper sulphate solution and alkaline tartrate solution are measured with a volumetric pipette and mixed. The copper sulphate solution must be made with great care, weighing the copper sulphate accurately. The urine is diluted by placing 10 cc. in a 100 cc. volumetric flask, adding water to bring the volume to 100 cc., and placed in a burette after thorough mixing. In a beaker, 10 cc. of Fehling's solution and 40 cc. of water are boiled over a flame. The burette is clamped over the beaker and when the Fehling's solution has reached the boiling-point, diluted urine is run from the burette until cupric oxide has been completely precipitated as red cuprous oxide, the end-point being indicated by disappearance of all blue color. The chief objection to the method is the uncertainty of the end-point. At least three determinations should be made, and the average taken of the readings.

Ten cubic centimeters (10 cc.) of Fehling's solution are reduced by 0.05 gm. dextrose (or 0.0676 gm. lactose). The fluid used for titration, therefore, contained 50 gm. of glucose (or 67.6 gm. lactose). If the dilution of urine were made as prescribed $(1+9)$, this formula may be employed for ascertaining percentage:

$50/X$ = percentage in original sample when X represents the number of cc. of diluted urine required to reduce the stated amount of reagent.

Preparation of Benedict's Reagent.—

Copper sulphate (crystallized)	18 gm.
Sodium carbonate (crystallized; one-half the weight of the anhydrous salt may be used)	200 "
Sodium or potassium citrate	200 "
Potassium thiocyanate	125 "
Potassium ferrocyanide (2 per cent. solution) .	5 cc.
Distilled water to make a total volume of	1000 "

With the aid of heat dissolve the carbonate, citrate and thiocyanate in enough water to make about 800 cc. of the mixture and filter if necessary. Dissolve the copper sulphate separately in about 100 cc. of water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool and dilute to exactly 1 liter in a volumetric flask. Of the various constituents, the copper salt only need be weighed with exactness. Twenty-five cc. of the reagent are reduced by 50 gm. of glucoso.

Procedure.—Ten cc. of urine are diluted in a volumetric flask to 100 cc. with distilled water, and a burette filled with the resulting mixture. Into a porcelain evaporating dish (about 25 to 30 cm. in diameter) 25 cc. Benedict's reagent are measured with a pipette, and 10 to 20 gm. crystallized sodium carbonate are added. One-half this amount should be used if the anhydrous salt be employed. A little powdered pumice or talc is added, and the mixture heated over a free flame until the carbonate has dissolved (Fig. 67). Then the diluted urine is quickly run in from the burette until the last trace of blue has gone. Throughout the titration the mixture must be kept boiling; should it become too concentrated, water may be added.

Calculation.—Twenty-five cc. of Benedict's reagent are reduced by 50 mg. glucose (67.6 mg. lactose, 74 mg. maltose, 47.5 mg. invert sugar). Therefore, the fluid used for titration contained 50 mg. of glucose. If the dilution of urine were made as prescribed (1+9), this formula may be employed for determining the percentage of glucose:

50/X = percentage in original sample when X represents the number of diluted urine required to reduce the stated amount of reagent.

Corresponding factors may be substituted for 50 with other sugars.

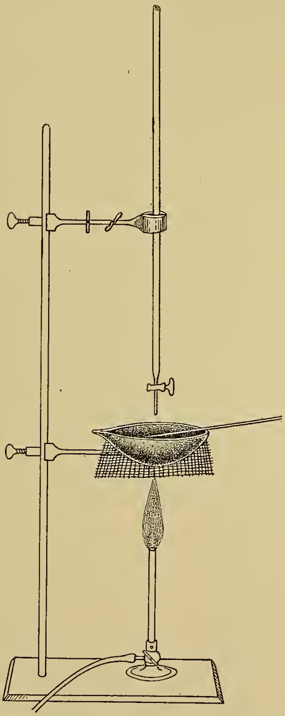


Fig. 67.—Arrangement of apparatus for quantitative determination of glucose by Benedict's method.

Benedict's Method Employing Small Quantities of Reagent.—V. C. Myers (personal communication) has suggested the use of smaller quantities of reagents. Five cc. of Benedict's solution are placed in a test-tube and about 2 to 3 gm. of anhydrous sodium carbonate added. The tube is held over the flame against a white background until the contents start to boil when urine is added from a Mohr pipette of 1 cc. capacity graduated in 0.1 ccs. The reagent is boiled gently. Meanwhile the urine is run in slowly until the end-point described above had been reached (Fig. 68). If the specific gravity be high, or if a preliminary determination show high sugar content, the urine should be diluted accordingly so that the amount of dilution required for reduction would be not less than 1 cc. At least two determinations should be made, and the average of the results taken. The method is readily performed in the simplest clinical laboratory and is extremely economical of reagents.

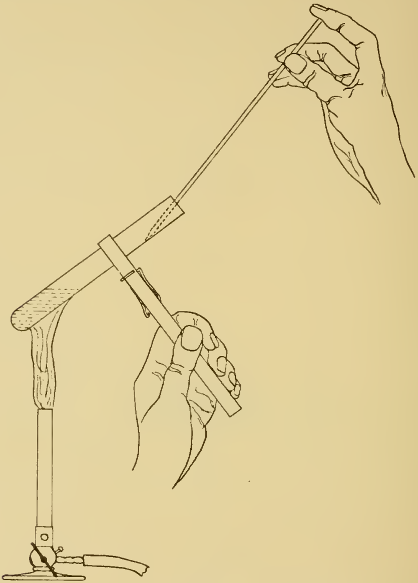


Fig. 68.—Determination of glucose by Myers's modification of Benedict's method.

Calculation.—Five cc. of Benedict's solution are reduced by 10 mg. of glucose. The amount of urine required to produce reduction, therefore, contained 10 mg. of glucose.

If the specimen were undiluted, 1/X gives the percentage of sugar in the undiluted urine when X represents the number of cc. of urine required to cause reduction. If the specimen were diluted the same formula would be used, X representing the number of cc. of diluted urine employed, and the result, of course, would be divided by the dilution.

Purdy's Method.—Preparation of Solution.—The solution is prepared as follows:

Pure cupric sulphate	4.752	gm.
Potassium hydroxid	23.500	“
Strong ammonia (U. S. P., sp. gr., 0.9)	350.000	cc.
Glycerol	38.000	“
Distilled water to	1000.000	“

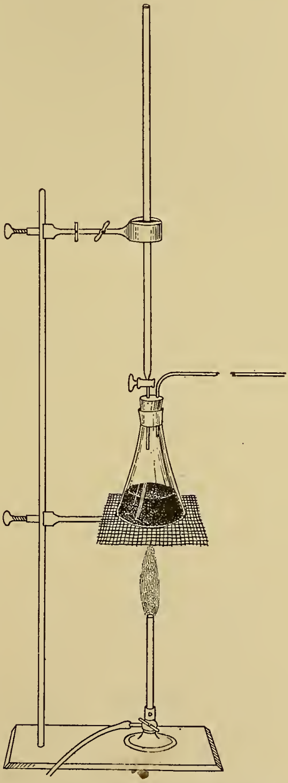


Fig. 69.—Arrangement of apparatus for determination of glucose by Purdy's method.

Prepare by dissolving the cupric sulphate and glycerin in 200 cc. of distilled water with the aid of moderate heat. In another 200 cc. of distilled water dissolve the potassium hydroxid. Mix the two solutions. Cool and add the ammonia. Finally, with distilled water bring the volume of the whole to exactly 1 liter in a volumetric flask.

Procedure. — Measure 35 cc. of Purdy's solution into an Erlenmeyer flask of about 250 cc. capacity, and add about 100 cc. of distilled water. Charge a 25 cc. burette, graduated in 0.1 ccs. with the urine, allowing it to run out until all the air in the terminal and rubber connections has been expelled. The Erlenmeyer flask should be provided with a tight-fitting rubber stopper, perforated with two holes (Fig. 69). The glass terminal of the burette is introduced through one hole, and through the other is passed a piece of glass tubing about 8 inches long, bent at right angles at a point a little above its emergence from the rubber stopper. This provides a vent to carry out the ammonia fumes. Mount the burette with attached Erlenmeyer flask and fittings on the burette stand, supporting the flask suitably with the iron ring, protecting the bottom with wire gauze. With a Bunsen flame, bring the contents of the flask to the boiling-point, and then slowly discharge urine from the burette until the blue color begins to fade; “then still more slowly, three to five seconds elapsing after each drop, until the blue color completely disappears and leaves the test-solution transparent and colorless." This marks the end-point. At least two determinations should be made, taking the average of the readings.

Calculation.—The amount of urine required to reduce 35 cc. of the solution contains exactly 0.02 gm. of sugar. The percentage can be determined with this formula:

$\frac{2}{X}$ = percentage of sugar, when X represents the number of cc. of urine required to effect reduction.

Example: 2 cc. of urine are required to reach the end-point. $2 \div 2 = 1$ per cent.

Precautions.—Should the amount of sugar exceed 3 to 4 per cent., it is well to repeat the manipulations with urine diluted with 2 or 3 parts of water. In this event the calculation should be corrected accordingly, dividing the result 3 or 4, as the case may be, before employing the formula given above.

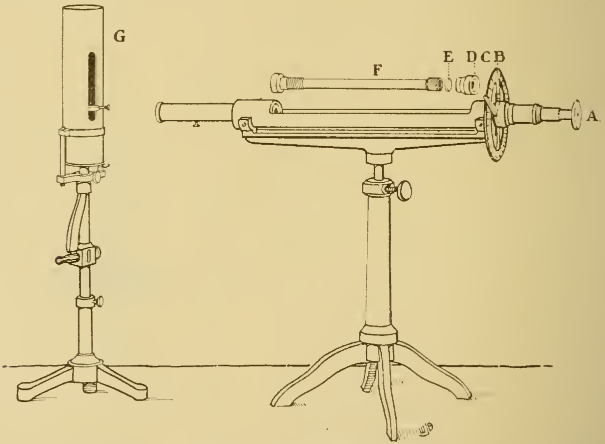


FIG. 70.—Polariscope. *A*, ocular; *B*, graduated dial; *C*, lever; *D*, cap; *E*, glass disk; *F*, tube for urine; *G*, lamp.

Quantitative Determination of Glucose by Polariscope. Urine containing albumin should be acidified with acetic acid, boiled to secure complete coagulation and filtered. It should be cleared by adding a few crystals of lead acetate and filtering, passing through the same filter two or three times if necessary to clarify completely. Before using the instrument the position of the zero mark should be adjusted. After the polariscope tube has been thoroughly cleaned with distilled water and dried perfectly it may be filled with cleared and filtered urine so that the meniscus is convex. The glass disk (Fig. 70) which serves as a cover for the end is slid on to displace any excess of fluid, leaving the tube full of urine and free from air-bubbles. The cap is screwed on and the tube placed in position in the instrument. Light should be obtained by vaporization of a sodium salt in the flame. It is convenient to employ a mixture made by fusing equal parts of sodium chloride and sodium phosphate, pulverizing the resulting mass. The handle of the instrument is rotated through 20 or 30 degrees to secure marked contrast between the two halves of the field and the eye-piece is focussed by drawing it out until a sharp line divides the two fields. The handle is then turned until the halves have the same intensity of brightness. Three or four readings should be made and the average taken, resting the eyes between readings.

For computing the amount of glucose the following formula may be used, in which G represents the grams of glucose in 100 cc. of urine; A the angle of rotation and L the length of the tube employed: The

$$G = \frac{A \times 100}{52.5 \times L}$$

tubes used for clinical purposes (polarizing saccharimeters) are made so that the readings may be made directly on the scale. With the short tube, 94.7 mm. long, each degree of rotation represents 2 per cent. of glucose, while with the long tube (189.4 mm. long) each degree represents 1 per cent. of glucose.

If a solution of glucose be prepared for testing the instrument it must be allowed to stand overnight, since it is birotary when first dissolved.

The specific rotation of the different sugars may be taken as dextrose, +52.5°; lactose, +53°; d-galactose, +81; levulose, −92°.

Chlorides.—*Simple Qualitative Test.*—If albumin be present, it should be removed by boiling a portion of the urine after acidifying it with acetic acid, and by filtering through paper. About 10 cc. of the filtrate should be employed. This should be strongly acidulated with nitric acid to prevent precipitation of the phosphates by the silver. About 5 drops of a 10 per cent. solution of silver nitrate are added. A similar quantity of normal urine should be treated in the same way as a control. When chlorides are present in the usual quantity, a heavy curdy precipitate appears and settles to the bottom of the tube quite promptly. When the chlorides are diminished in quantity, the precipitate will be extremely light. This qualitative test is sufficient for the mere determination of an increase or diminution of the quantity of chlorides.

Principle.—The chlorides are precipitated by silver nitrate, potassium chromate serving as an indicator of complete precipitation. When the chlorides have been precipitated completely, the silver nitrate is free to combine with the chromate radical, forming silver chromate, which is orange in color.

Reagents Required.—(1) *Standard Silver Nitrate Solution.*—This should contain 29.059 gm. of pure silver nitrate dissolved in a liter of water. It should be prepared and standardized according to the method described in the following section. (2) Five per cent. potassium chromate.

Procedure.—The urine is freed from albumin as described in the foregoing section (simple qualitative test). Ten cc. of the filtrate are placed in a porcelain evaporating dish with about 100 cc. of water. Five drops of 5 per cent. potassium chromate solution are added and the standard silver nitrate solution is run in from a burette until the color of the mixture in the evaporating dish becomes distinctly orange in color. The end-point is the first appearance of a definite and permanent orange-red. The mixture in the evaporating dish should be stirred constantly while adding the silver nitrate. A second determination should be made with a second 10 cc. quantity and the average of the two readings should be taken.

Calculation.—One cc. of standard silver nitrate solution is precipitated by 0.01 gm. of sodium chloride. The number of cc. of silver solution required for neutralization of 10 cc. multiplied by 0.01 gives the chlorides in 10 cc. of urine. This result divided by 10 gives the amount per cc.

Harvey's Modification of Volhard's Method.—*Principle.*—The chlorides are precipitated by a stipulated quantity of a standard solution of silver nitrate in the presence of nitric acid, and the amount of unprecipitated silver nitrate determined by titration with a standard solution of ammonium sulphocyanate, using a solution of iron alum as an indicator. When all of the silver has combined with the sulphocyanide to form silver sulphocyanate, additional ammonium sulphocyanate forms ferric sulphocyanate, which gives a yellow color and indicates the end-point.

Reagents Required.—1. Silver nitrate solution, of which 1 cc. precipitates 0.01 gm. of NaCl. For this 29.059 gm. of silver nitrate should be dissolved in 1 liter of water. This solution may be checked as follows: Chemically pure NaCl is dried at 120° C. for two hours. Then 0.150 gm. is weighed out and dissolved in 100 cc. of distilled water. Five drops of 5 per cent. potassium chromate are added and the silver solution is added from a burette. It should require 15 cc. of the latter to produce an orange tint.

2. Ammonium sulphocyanate solution, of which 1 cc. precipitates 1 cc. of the silver nitrate solution as described under 1. Since the salt absorbs water, it cannot be weighed with exactness. Thirteen grams are dissolved in 800 cc. of water. This solution is then titrated against the silver nitrate solution. Ten cc. of the silver nitrate solution are placed in an evaporating dish with 100 cc. of water. The mixture is acidified with HNO_3 and 2 cc. of the indicator solution are added. The ammonium sulphocyanate solution is then run in from a burette until a permanent red color is secured. If the solution be correctly

prepared, exactly 10 cc. would be required. If less than this amount were needed, the solution was too strong and will require dilution with water; if more than 10 cc. were used, the solution was too weak and will require additional ammonium sulphocyanate. The exact amount may be computed mathematically, but after readjustment the solution must be titrated until its strength is such that exactly 10 cc. precipitate 10 cc. of the silver solution.

3. Indicator solution, made by dissolving 100 gm. of ferric ammonium sulphate in 100 cc. of 25 per cent. nitric acid.

Procedure.—Measure 5 cc. of urine, freed from albumin, into a white porcelain evaporating dish. Add 20 cc. distilled water, 10 cc. standard silver solution, and 2 cc. of indicator solution. Mix thoroughly and run in standard ammonium sulphate solution from a burette until the first trace of yellow shows through the mixture.

Calculation.—Subtract the number of cc. of sulphocyanate solution used in titration from 10 and multiply by 0.01 to determine the number of gm. of sodium chloride in 5 cc. urine, or by 0.20 to determine the gm. per 100 cc.

Normal Quantity.—Under normal conditions, the amount of chlorides voided in twenty-four hours is between 10 and 15 gm.

Significance.—The regulation of normal metabolism requires a chloride equilibrium, a definite amount being retained by the tissues of the body for the maintenance of osmotic equilibrium. Ingestion of greater amounts of chloride than usual, may be followed by temporary retention but the excess is eliminated rather rapidly. Similarly a diminution in the intake of chlorides (as in starvation) results in a diminished chloride excretion, since the chlorides are held by the body to maintain a suitable chloride balance. The amount of chlorides excreted is diminished during the course of febrile diseases, where there are large exudates, in severe diarrhea, in pneumonia, and after inhalation of chloroform. The chloride excretion increases in amount during and after the crisis in pneumonia or when an exudate is absorbed. The chloride equilibrium is disturbed in advanced chronic nephritis with edema, the chlorides being retained. Attempts have been made to use chloride excretion as a prognostic guide in pneumonia, but without success.

Determination of Phosphates and Sulphates.—The determination of these substances has value only in metabolic work, and methods for making these determinations may be obtained from standard texts on physiological chemistry. Determinations made on a single specimen, without regard to the intake and to the analysis of feces, have no value whatever. The clinical value of the results of phosphate and sulphate determination, does not justify the description of the technic in this book.

Total Nitrogen.—*Principle.*—By boiling urine with concentrated sulphuric acid in the presence of an oxidizing agent, the nitrogenous bodies are converted to ammonium sulphate. This is broken down by the action of an alkali with the formation of ammonia, which is liberated by distillation and is collected in acid of known strength. The acid may then be titrated with a standard alkali solution, and the amount of liberated and neutralized ammonia determined.

Reagents Required.—(1) Crystals of copper sulphate. (2) Concentrated sulphuric acid. (3) Potassium sulphate. (4) Powdered pumice. (5) Paraffin. (6) Decinormal sulphuric acid. (7) Congo-red solution. (8) Decinormal sodium hydrate.

Procedure.—In a long-necked Kjeldahl flask should be placed 5 cc. of urine, measuring the quantity with volumetric pipette. A crystal of copper sulphate and 20 cc. of concentrated sulphuric acid should be added. The flask is heated over wire-gauze in a fume-closet until white fumes are driven off, when 2 gm. of potassium sulphate are added. This aids the process of decomposition by raising the boiling-point of the sulphuric acid. The mixture should be boiled for at least thirty minutes after it has become colorless. The flask is cooled and 300 cc. of ammonia-free distilled water are added, with a teaspoonful of powdered pumice to prevent bumping, a small piece of paraffin to prevent frothing, and 60 cc. of 30 per cent. sodium hydrate to alkalinize the mixture. More than this quantity of NaOH should be used if needed to accomplish the result. The alkali should not be allowed to touch the neck of the flask, since its presence here would interfere with the tight-fitting of the rubber stopper. The mixture is shaken and is ready for distillation (Fig. 71). The flask is connected with the safety-bulb of the apparatus, and the tip of the glass safety-tube as it

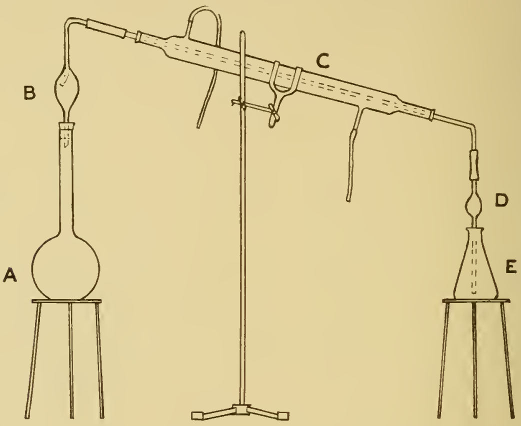


FIG. 71.—Kjeldahl distilling apparatus. *A*, flask containing digested urine; *B*, safety bulb; *C*, Liebig condenser; *D*, safety bulb; *E*, flask for receiving distillate.

emerges from the condenser is placed under the level of the liquid in an Erlenmeyer flask of 500 cc. capacity, in which should have been placed 50 cc. of decinormal sulphuric acid with ten drops of Congo-red solution and about 100 cc. of distilled water. Distillation should be conducted for at least an hour, starting with a small flame. At the end of this time, a drop of the distillate should be tested with litmus paper as it drops from the tip of the safety-tube. If still alkaline, distillation should be continued until the distillate no longer turns red litmus-paper blue. When the fluid from the condenser is no longer alkaline, boiling is discontinued and the fluid in the Erlenmeyer flask is titrated by adding decinormal sodium hydrate from a burette until the fluid shows a brilliant red color.

When reagents of unknown quality are being employed, a blank determination should be run through, omitting only the urine, and the result obtained should be subtracted as a correction from that secured with regular determinations.

Résumé of Procedure.

DIGESTION.

1. Place 5 cc. of urine in long-necked Kjeldahl flask.
2. Add (*a*) 20 cc. of concentrated sulphuric acid.
    (*b*) A crystal of copper sulphate.
3. Heat until white fumes appear.
4. Add 2 gm. of potassium sulphate.
5. Boil for thirty minutes after mixture becomes colorless.
6. Cool flask.

DISTILLATION.

7. Add (*a*) 300 cc. of distilled water.
    (*b*) Teaspoonful of powdered pumice.
    (*c*) Small piece of paraffin.
    (*d*) 60 cc. of 30 per cent. sodium hydrate, or more if needed to alkalinize.
8. In Erlenmeyer flask place:
    (*a*) 50 cc. of $\frac{N}{10}$ H_2SO_4.
    (*b*) 100 cc. of distilled water.
    (*c*) 10 drops of Congo-red solution.
9. Start distillation with low flame, continuing for at least an hour, until the distillate fails to give alkaline reaction with litmus.

TITRATION.

10. Titrate contents of Erlenmeyer flask with $\frac{N}{10}$ NaOH until brilliant red color is obtained.
11. Make calculation as shown below.

Calculation.—Subtract the number of cubic centimeters of $\frac{N}{10}$ NaOH used in titration from the number of cubic centimeters of $\frac{N}{10}$

H_2SO_4 in the Erlenmeyer flask. The difference equals the number of cc. of $\frac{N}{10}$ H_2SO_4 neutralized by the liberated ammonia. Since 1 cc. of $\frac{N}{10}$ H_2SO_4 is equivalent to 0.0014 gm. of nitrogen, the total amount of nitrogen is equal to the number of cc. of $\frac{N}{10}$ H_2SO_4 neutralized multiplied by 0.0014. The result represents the amount in 5 cc. of urine. The percentage can be ascertained by multiplying the number of cc. of $\frac{N}{10}$ H_2SO_4 neutralized by the factor 0.028.

Colorimetric Method (Myer's Modification of Folin's Method).—This method is to be given preference for clinical work.

Principle.—A small quantity of urine is treated with sulphuric acid in the presence of an oxidizing agent and the nitrogenous bodies are converted to ammonium sulphate, as in the preceding method. After dilution the residue is Nesslerized and compared colorimetrically with a standard solution, similarly Nesslerized, containing a known quantity of nitrogen in the form of ammonium sulphate.

Reagents Required.—(1) Crystals of copper sulphate. (2) Crystals of potassium sulphate. (3) Sulphuric acid (C. P.). (4) Hydrogen peroxide. (5) Standard solution of ammonium sulphate, 5 cc. containing 1 mg. of nitrogen (6) Nessler's solution. For the preparation of the two latter reagents, see pages 177 and 178.

Procedure.—To 5 cc. of urine are added 45 cc. of water, making a 1 : 10 dilution. One cc. of the diluted urine is placed in a thick-walled test-tube and to it are added one drop of 10 per cent. copper sulphate solution, a crystal of potassium sulphate, and 0.1 cc. of sulphuric acid, C. P. The tube is held in a clamp attached to a tripod and heat is applied with a micro-burner, first to remove the water and then to digest the nitrogenous compounds with the sulphuric acid. Provision must be made for carrying off the fumes (Fig. 56). Heating is continued for two minutes after the mixture becomes colorless. If a clear blue color does not develop readily, a drop or two of hydrogen peroxide is added while the mixture is still warm. It is then allowed to cool somewhat though not to the point of solidification.

Two 100 cc. cylindrical graduates should be in readiness. A 20 per cent. dilution of Nessler's solution is made up with distilled water. The contents of the tube are dissolved quickly in about 10 cc. of water and the solution is poured into the graduate, rinsing the tube into the graduate with a little distilled water to bring the total volume to 15 cc. In the second 100 cc. graduate is placed 5 cc. of the standard solution of ammonium sulphate (5 cc. of which contains 1 mg. of nitrogen) and to it is added 20 cc. of 20 per cent. Nessler's solution and distilled water to bring the volume up to 100 cc. To the unknown, a sufficient quantity of the 20 per cent. Nessler's solution is added quickly to secure the maximum amount of red coloration, when the mixture is diluted with water to make the total volume either 50 or 100 cc., whichever amount is required to make the color of the diluted unknown most closely approximate the color of the standard. A portion of the standard is then placed in the left-hand cup of the colorimeter and the cup is set at a depth of 10 mm.; this is moved up and down until the color in the two halves of the visual field match.

Résumé of Procedure.

1. Make a 1 : 10 dilution of urine.
2. Place 1 cc. of dilution in a thick-walled test-tube.
3. Add the following:
    - (*a*) 1 drop of 10 per cent. copper sulphate.
    - (*b*) Crystal of potassium sulphate.
    - (*c*) 6 drops of sulphuric acid, C. P.
4. Place tube in clamp and heat with micro-burner until mixture becomes colorless.
5. If blue color does not appear, add a drop or two of hydrogen peroxide.
6. Cool, but not to the point of solidification.
7. Take two graduated cylinders of 100 cc. capacity and handle as follows, placing digested urine mixture in cylinder *B*.

CYLINDER A.	CYLINDER B.
8. Place in cylinder 5 cc. of standard ammonium sulphate solution, containing 1 mg. of nitrogen.	Digest mixture with 10 cc. of water.
9. Add 20 cc. of 20 per cent. Nessler's solution.	Add 20 per cent. Nessler's solution to secure maximum color.
10. Add water to make total volume 100 cc.	Add water to make color approximately same as solution in *A*.
11.Compare in colorimeter.	

Calculation.—Let R represent the reading of the unknown when the standard is set at ten. When the unknown is diluted to 100 the number of milligrams of nitrogen per cc. of undiluted urine can be ascertained by the following equation : Mg. nitrogen per cc. $= \frac{100}{R}$. If the unknown were diluted to only 50 cc. the result should be divided by 2. The same figure also represents the gms. per liter. The percentage is represented by placing a decimal point in front of the figure ascertained.

Normal Quantity.—In health on an average diet the total amount of nitrogen excreted in the urine in twenty-four hours is about 16 to 18 gm.

Urea.—*Principle.*—Urea is converted to ammonium carbonate by urease, an enzyme found in certain fungi, bacteria, and higher forms of plant life. By comparing the alkalinity of urine treated with urease with that of an untreated specimen of equal volume, the amount of urea may be computed. Urease for biochemical purposes is ordinarily obtained from the soy bean (glycerine hispida) or from the jack bean.

Urease.—It is desirable to obtain urease in the form of a dry and readily soluble powder, prepared with potassium dihydrogen phosphate and dipotassium hydrogen phosphate so combined that the mixture will be exactly neutral on solution. The neutral phosphate mixture serves as a stabilizer for the enzymes. Urease in this form is prepared by the Arlington Chemical Company, Yonkers, N. Y., and by E. R. Squibb and Sons. Compressed tablets of urease are put on the market by Hynson, Westcott and Dunning, Baltimore, Md. The activity of a particular lot of urease should be determined. Usually a two and one-half to five per cent. of the Squibb preparation and a ten per cent. solution of the Arlington preparation may be made.

Three methods employing this principle are available. The extreme simplicity of Marshall's method commends it to the practitioner.

Determination by Direct Titration of Urine Treated with Urease (Marshall's Method).—*Procedure.*—This is the simplest method. Two Erlenmeyer flasks of about 200 cc. capacity are taken. Into each is placed 1 cc. of the urine to be examined. In one flask is placed urease, either 2 cc. of the freshly prepared solution or two tablets (as prepared by Hynson, Westcott and Dunning). The latter should be thoroughly crushed with a little water before being added. Enough distilled water should be added to each flask to bring the total volume up to about 100 cc. The two flasks should then be subjected to a temperature of 40° C. for fifteen minutes, when into each flask should be placed 5 drops of 1 per cent. alcoholic solution of methyl orange. $\frac{N}{50}$ HCl is added from a burette until the color becomes distinctly pink.

Calculation.—The amount of ammonium carbonate formed is indicated by the increased alkalinity of the specimen treated with urease compared to the control specimen. From the number of cc. of $\frac{N}{50}$ HCl required to neutralize the specimen treated with urease, subtract the number of cc. required to neutralize the control, and multiply the remainder by the factor 0.6. The result will give the number of gm. of *urea* per 1000 cc. of urine. The amount of *urea nitrogen* may be computed by dividing this figure by the factor 2.14.

Determination by Aspiration and Titration of the Liberated Ammonia after Treatment of the Urine with Urease (Van Slyke and Cullen).—Urine is treated with urease as in the Marshall method and the ammonium carbonate is broken down to ammonia by the addition of a strong alkali. The liberated ammonia is then aspirated by an air current into a measured quantity of a standard acid solution, by which it is neutralized. The amount so liberated and neutralized may be determined by titrating the acid with a standard alkaline solution.

In this method, the urinary ammonia is determined as well as the amount of ammonia formed from urea. It is necessary, therefore, to ascertain the amount of urinary ammonia by the same method, treating a similar quantity of urine with alkali and aërating but omitting the treatment with urease. The amount of urinary ammonia is subtracted from the amount of urinary ammonia plus urea to give the amount of urea.

Reagents Required.—Solution of urease: Amyl or caprylic alcohol: $\frac{N}{50}$ HCl; 1 per cent. solution of sodium alizarinate; $\frac{N}{50}$ sodium hydrate; saturated solution of sodium carbonate.

Procedure.—The urine is diluted by adding 45 cc. of water to 5 cc. of urine. Into cylinder *A* of the aërating device (Fig. 55) there should be measured 5 cc. of the *diluted* urine, 2 cc. of urease solution, and a drop of caprylic or amyl alcohol to prevent bumping. The stopper is closed and the cylinder is placed in a water-bath at a temperature of 40° C. for fifteen minutes to allow the enzyme to act. Meanwhile 25 cc. of $\frac{N}{50}$ HCl (or H_2SO_4) are placed in cylinder *B* with one drop of 1 per cent. sodium alizarinate indicator and one drop of caprylic or amyl alcohol. When fifteen minutes have expired, *A* and *B* in series are connected and the air current *B* allowed to pass for a half minute, in order to drive over into the acid in *B* any ammonia which may have escaped into *A*. The air should be drawn through a wash-bottle containing dilute sulphuric acid to remove any contained ammonia. At the end of the preliminary aspiration, cylinder *A* is opened and 5 cc. of a saturated solution of sodium carbonate are added. *A* is again closed and the air current is allowed to pass until all the ammonia is exhausted from *A*. The time required will vary with the efficiency of the pump or pressure system which is used, ranging from five to thirty minutes. When aspiration is completed, the tube in *B* is washed into *B* with a small quantity of distilled water and $\frac{N}{50}$ sodium hydrate is added to the contents of *B* until a purple color appears.

RÉSUMÉ OF METHOD.

1. Make a 1 : 10 dilution of urine.
2. Place 5 cc. of this dilution in cylinder *A*.
3. Add
    - (*a*) 2 cc. of 20 per cent. urease solution.
    - (*b*) A drop or two of amyl or caprylic alcohol.
4. Stopper *A* and place in water-bath at 40° C. for fifteen minutes.
5. In *B* place
    - (*a*) 25 cc. of $\frac{N}{50}$ HCl.
    - (*b*) A drop of amyl or caprylic alcohol.
    - (*c*) A drop of 1 per cent. sodium alizarinate.
6. Connect *A* and *B* in series, turn on air current and allow it to pass about one-half minute.
7. Disconnect, add 5 cc. of saturated solution sodium carbonate to *A*.
8. Reconnect *A* and *B* and allow air current to pass about thirty minutes.
9. Wash absorption tube in *B* into cylinder *B* with water.
10. Titrate contents of *B* using $\frac{N}{50}$ NaOH until purple color appears.
11. Make calculation as shown below.

Calculation.—The number of cc. of fiftieth-normal acid neutralized by the liberated ammonia is computed by subtracting from 25 the number of cc. $\frac{N}{50}$ sodium hydrate used in the titration. This is multiplied by the factor 0.56 to give the number of gm. of *urea plus ammonia nitrogen* in 1000 cc. of urine. To ascertain the number of gm. *urea nitrogen,* determine the amount of *ammonia nitrogen* and subtract. The amount of urea may be calculated from the urea nitrogen by multiplying by the factor 2.14.

Myers' Colorimetric Method.—This is the preferred method.

Reagents Required.—(1) Solution of urease. (2) Amyl or caprylic alcohol. (3) $\frac{N}{50}$ HCl. (4) Saturated solution of sodium carbonate. (5) Nessler's solution. (6) Standard solution of ammonium sulphate, 5 cc. containing 1 mg. of nitrogen. (For the preparation of the latter two reagents, pages 177 and 178.)

Procedure.—Two cc. of a 1 : 10 dilution of urine are placed in a test-tube with 1 cc. of urease solution (10 per cent. in strength if the Arlington brand be employed) and a drop of two of amyl or caprylic alcohol. The tube is stoppered and placed in a water-bath at 40° C. for fifteen minutes as in the preceding method. Aëration is conducted also as was directed before, the test-tube being placed in cylinder *A*, cylinder *B* being charged with 25 cc. of $\frac{N}{50}$ HCl acid (Fig. 55). Before the air current is started, 5 cc. of a saturated solution of sodium carbonate should be added to the contents of the tube in cylinder *A*. Aëration is allowed to proceed for about thirty minutes. The contents of the test-tube are placed in a 100 cc. graduated cylinder. The tube is rinsed with a few cc. of distilled water which are poured into the graduate also, and freshly diluted 20 per cent. Nessler's solution is added until there is no further deepening of the color. Distilled water is added to make the total volume 50, 100, or 200 cc., whichever may be necessary to most nearly approximate the color of the standard.

The standard is prepared simultaneously by putting in a similar cylindrical graduate 5 cc. of the ammonium sulphate standard (5 cc. containing 1 mg. of nitrogen), 10 cc. of water, 20 cc. of the freshly diluted 20 per cent. Nessler's solution, and distilled water in sufficient quantity to make the total volume 100 cc.

RÉSUMÉ OF METHOD.

1. Make a 1 : 10 dilution of urine.
2. Place 2 cc. in a test-tube.
3. Add
    (*a*) 1 cc. of urease solution.
    (*b*) A drop or two of amyl or caprylic alcohol.
4. Stopper tube and place in water-bath at 40° C. for fifteen minutes.
5. In cylinder *B* place:
    (*a*) 25 cc. of $\frac{N}{50}$ HCl.
    (*b*) A drop or two of amyl or caprylic alcohol.

6. Place test-tube in cylinder *A* and remove stopper.
7. Add 5 cc. saturated solution of sodium carbonate.
8. Connect cylinders *A* and *B*, turn on air-current. Allow air to pass for about thirty minutes.
9. Wash out absorption tube in *B* into cylinder *B* with 2 or 3 cc. of water.
10. Treat contents of cylinder *B* as shown below, making up a standard for comparison in cylinder *C*.

CYLINDER B.	CYLINDER C.
11.	Place in cylinder 5 cc. of ammonium sulphate standard (contains 1 mg. of nitrogen).
12. Add 20 per cent. dilution of Nessler's solution until no further deepening of color.	Add 20 cc. of 20 per cent. solution of Nessler's solution.
13. Add distilled water to 50, 100, etc., cc., to match color of *C* approximately.	Add distilled water to make volume 100 cc.

14. Compare in colorimeter.

Calculation.—The results may be arrived at by dividing 50 by the reading when the standard is set at 10. If the dilution were made to 100, the result is the number of gm. of *urea and ammonia* nitrogen per cc. of urine. If the dilution were to 50, the result should be divided by 2, and if to 200, it should be multiplied by 2.

Normal Quantity.—The amount of urea nitrogen excreted in twenty-four hours by a normal individual on an average diet is 13 to 16 gm. Of the total nitrogen excreted, from 86 to 90 per cent. is urea nitrogen.

Ammonia Nitrogen.—The determination of ammonia may be performed at the same time that the urea determination is made, setting up the two cylinders necessary for aspiration in series with those used for the urea determination. When the amount of ammonia nitrogen has been ascertained, it may be subtracted from the amount of urea and ammonia nitrogen, and the remainder will represent the urea nitrogen. This may be translated to terms of urea by multiplying by the factor 2.14.

Ammonia.—*Determination by Aspiration and Titration* (Folin's Method.)—This is the simpler method.

Reagents Required.—See determination of urea by aspiration and titration. Urease is not required.

Principle.—The principle and procedure is the same as that for the determination of urea by aspiration and titration except that urease is not used. Five cc. of urine are placed in cylinder *A* (Fig. 55) of the aspiration cylinder, with a drop or two of amyl or caprylic alcohol. In cylinder *B* should be placed 25 cc. of $\frac{N}{50}$ HCl (or H_2SO_4), with a drop of 1 per cent. sodium alizarinate and a drop or two of amyl or caprylic alcohol. In cylinder *A* are put 5 cc. of a saturated solution of sodium carbonate. The cylinders are connected and the air current is allowed to pass through for the requisite length of time. For details regarding aëration, see the application of the same method to the determination of urea and ammonia nitrogen in the preceding paragraphs. When the aëration is complete, the absorption-tube in *B* is washed into *B* with a little distilled water and $\frac{N}{50}$ NaOH is added from a burette to the contents of *B* till a purple color appears.

Calculation.—From 25 is subtracted the number of cc. of $\frac{N}{50}$ NaOH required to neutralize the acid. The difference represents the number of cc. of acid neutralized by liberated ammonia. Multiplying this by the factor 0.056 will give the number of grams of ammonia *nitrogen* per 1000 cc. of urine. The ammonia may be determined by multiplying the ammonia nitrogen by the factor 1.21.

Colorimetric Method.—Reagents Required.—As for the determination of urea by the colorimetric method, except that urease is not needed.

Procedure.—Undiluted urine is used. The determination is performed in the same manner as described for the determination of urea and ammonia nitrogen in the preceding section (see page 258) except of course that urease is not added to break down urea into ammonium carbonate. Two cc. of undiluted urine are placed in cylinder *A* of the aëration outfit and cylinder *B* is charged with 25 cc. $\frac{N}{50}$ HCl. Five cc. of a saturated solution of sodium carbonate are added to the urine in test-tube *A*, which is then placed in the cylinder, the cylinders are stoppered, and the air current allowed to run through until the ammonia has been completely removed. Nesslerization is conducted as described in the preceding section for the determination of urea and ammonia nitrogen, and the colorimetric reading and calculation is made in the same way. The result gives the number of grams of ammonia nitrogen per cc. of urine. Multiplying this by the factor 1.21 will give the number of grams of ammonia per cc. of urine.

Folin's Alternate Clinical Method.—Principle.—The acidity of a given quantity of urine is determined by titration with $\frac{N}{10}$ NaOH. To the urine is then added neutralized formalin, which combines with the ammonia to form neutral hexamethylene-tetramin and so sets free the acid equivalent of the amount of ammonia present.

Reagents Required.—(1) $\frac{N}{10}$ NaOH solution. (2) phenolphthalein solution. (3) a saturated solution of potassium oxalate solution (neutral to phenolphthalein). (4) formalin, also neutral to phenolphthalein.

The two latter reagents are neutralized by adding a few drops of the phenolphthalein solution and then enough $\frac{N}{10}$ NaOH to produce a faint pink coloration.

Procedure.—Place in a flask 5 cc. of the neutralized potassium oxalate solution, 3 drops of phenolphthalein solution, and 25 cc. of urine. Titrate with $\frac{N}{10}$ NaOH to produce a faint but unmistakable pink color. Then add 5 cc. of neutralized formalin and again titrate to the same pink color. The difference between the total amount of alkali employed and that used for the first titration represents the alkali required to neutralize the acid set free by the combination of ammonia with formaldehyde. Each cc. of $\frac{N}{10}$ alkali used corresponds to a cc. of $\frac{N}{10}$ ammonia.

Calculation.—This should be carried out as in the previous test.

Normal Quantity.—In health with an average diet the amount of ammonia nitrogen excreted in twenty-four hours is about 0.7 gm. The ammonia nitrogen represents from 3.3 to 5 per cent. of the total nitrogen excreted. The amount is greatly increased in acetone acidosis.

Determination of Uric Acid.—*Colorimetric Method.*—(Benedict and Hitchcock's modification of Folin and Denis' method.)

Principle.—The purins are precipitated by an ammoniacal silver-magnesia mixture. The precipitate is separated by removing the ammonia and then is dissolved with hydrocyanic acid. In the presence of the phosphotungstic acid of the Folin-Denis reagent a color reaction is obtained, which may be used as a quantitative test by employing a comparison tube containing a known quantity of uric acid, similarly treated.

Reagents Required.—(1) Saturated solution of sodium carbonate. (2) 5 per cent. solution of sodium or potassium cyanide. (3) Folin-Denis reagent (for preparation of this reagent, see page 191). (4) ammoniacal silver-magnesium mixture.

For this it is necessary to make a magnesia mixture first, which is prepared by dissolving 36 gm. of magnesium sulphate and 70 gm. of ammonium chloride in 280 cc. of distilled water and then adding 140 cc. of concentrated ammonia.

The ammoniacal silver-magnesia solution is prepared according to the following formula:

3 per cent. silver nitrate solution	70 cc.
Magnesia mixture	30 "
Concentrated ammonia	100 "

If turbidity develops, the mixture should be filtered.

Procedure.—In a graduated 15 cc. centrifuge tube with conical tip (Fig. 54, *g*) are placed 2 cc. of urine and 3 cc. of water. To this are added 15 drops of silver magnesium mixture. To cause complete precipitation of the purins, the tube is placed in a beaker containing cracked ice and salt, where it is allowed to remain for fifteen minutes. It is then centrifugalized and the supernatant fluid is decanted off. The tube is stood upside down to drain off the residual fluid and the ammonia is gotten rid of by introducing well into the tube a piece of glass tubing attached to a suction pump.

To the precipitate in the tube are added 2 or 3 cc. of water and 2 drops of a 5 per cent. solution of potassium (or sodium) cyanide. A fine glass rod should be used to stir the sediment and to secure complete solution. Two cc. of Folin-Denis reagent are then introduced and the contents of the tube are poured into a 100 cc. cylindrical graduate, rinsing the centrifuge tube into the graduate with a saturated solution of sodium carbonate, about 20 cc. carbonate solution are employed.

The standard is prepared by placing in a similar cylindrical graduate of 100 cc. capacity, 5 cc. of the uric acid standard (prepared so that it will contain 1 mg. of uric acid), 2 drops of a 5 per cent. solution of potassium (or sodium) cyanide, 2 cc. of Folin-Denis reagent, 20 cc. of saturated solution sodium carbonate, and distilled water to make the total volume 100. Water should now be added to the graduate containing the unknown to 50, 100, or even 200 cc., as may be necessary to make the colors in the two graduates of approximately the same depth. In making up the standard and the unknown it is well to carry out the various steps as nearly simultaneously as possible.

Résumé of Method.

1. Place 2 cc. of urine in graduated centrifuge tube.
2. Add (*a*) 3 cc. of distilled water.
   (*b*) 15 drops of ammoniacal silver magnesia mixtures.
3. Stand tube in mixture of cracked ice and salt for fifteen minutes.
4. Centrifugalize.
5. Decant off supernatant fluid and discard it.
6. Drain fluid from tube and aspirate the ammonia.
7. Treat precipitate of uric acid in tube as shown below, preparing standard at the same time.

UNKNOWN.	STANDARD.
8. Add 3 cc. water to sediment in the tube.	Place in 100 cc. cylinder 5 cc. of uric acid standard solution containing 1 mg. of uric acid nitrogen.
9. Add 2 drops of 5 per cent. sodium cyanide solution.	Add 2 drops of 5 per cent. sodium cyanide solution.
10. Add 2 cc. of Folin-Denis reagent.	Add 2 cc. of Folin-Denis reagent.
11. Pour into 100 cc. cylinder and rinse tube into cylinder with saturated solution of 20 cc. of sodium carbonate.	Add 20 cc. saturated solution of sodium carbonate.
12. Add water to 50, 100, or 200 cc. mark to approximately match color of standard.	Add water to make volume 100 cc.

13. Compare contents of two cylinders in colorimeter.

Calculation.—When the unknown is diluted to 100 cc., and the cup of the colorimeter containing the standard is set at ten, the figure 5 may be divided by the reading of the unknown. This will give the number of gm. of uric acid nitrogen in 1000 cc. of urine.

When the unknown has been diluted to 50 cc., the same formula may be used, but the final result should be divided by 2, while if it were diluted to 200, the result should be multiplied by 2.

Normal Findings.—Under conditions of health with an average diet, the amount of uric acid nitrogen ranges from 0.08 to 0.15 gm. per diem. The average output makes up from 0.6 to 1.0 per cent. of the total nitrogen.

Determination of Creatine and Creatinine (Folin's later method, Folin and Morris).

Principle.—Folin has raised the objection that his older colorimetric method, which employed a bichromate standard, imposed an unnecessary limitation upon a flexible method since the results were unsatisfactory with small amounts of creatinine. He therefore, modified this method so as to employ a creatinine standard, the urine and the standard being subjected to the same treatment with alkali in the presence of picric acid. The principle is the same as that underlying the determination of creatinine in the blood, relying upon the power of creatinine to reduce sodium picrate.

Reagents Required.—(1) Standard creatinine solution, containing 1 mg. of creatinine in 1 cc. of a saturated solution of picric acid (see page 444). (2) 10 per cent. solution of sodium hydrate.

Procedure for Creatinine.—One cc. of the standard creatinine solution (containing 1 mg. of creatinine) is placed in a 100 cc. volumetric flask, and 1 cc. of urine is placed in another 100 cc. volumetric flask. To each flask are then added 20 cc. of saturated picric acid solution measured with a volumetric pipette, and 1.5 cc. of 10 per cent. sodium hydrate solution, measured with a burette or with a pipette graduated in 0.1 cc. At the end of ten minutes the flasks are filled to the 100 cc. mark with distilled water.

Résumé of Method.

FLASK A.	FLASK B.
1. Place in flask 1 cc. of urine.	Place in flask 1 cc. of standard creatinine solution (1 mg. of creatinine).
2. Add 20 cc. of saturated solution of picric acid.	Add 20 cc. of saturated solution of picric acid.
3. Add 1.5 cc. of 10 per cent solution of sodium hydrate.	Add 1.5 cc. of 10 per cent solution of sodium hydrate.
4. Allow to stand for ten minutes.	Allow to stand for ten minutes.
5. Add distilled water to 100 cc. mark.	Add distilled water to 100 cc. mark.
6. Compare a portion of contents of two flasks in colorimeter.	

Calculation.—When the standard has been set at 10 mm., ten is divided by the reading of the unknown. The results give the mg. of creatinine in the volume of urine employed in making the determination. If the urine reads less than two-thirds or more than one and a half that of the standard, the determination should be repeated with more or with less urine, as the case may be, so that the amount of creatinine in the unknown will be approximately that in the standard.

Procedure for Creatine.—Creatine is broken down to creatinine by prolonged boiling with picric acid and the total creatinine is then determined. From this the amount of preformed creatinine, which has been determined previously, may be subtracted.

Into a weighed Erlenmeyer Pyrex flask of 200 cc. capacity is placed enough urine to yield about 0.7 to 1.5 mgm. of creatinine when the creatine is broken down. With this is placed 20 cc. of a saturated solution of picric acid and 130 cc. of distilled water. A few small pebbles are used to prevent bumping and the mixture is boiled gently over a microburner for about an hour. Toward the end of the time, the heat is increased so that the solution may be boiled down to somewhat less than 20 cc. The flask is placed upon the scales and enough water added to bring the weight of the contained fluid to 20 to 25 gm. The flask and its contents are then cooled in running water and 1.5 cc. of 10 per cent. NaOH are added, the total volume is brought up to 100 cc. with distilled water, and the amount of creatinine is determined by using a standard of known strength, as described in the previous section.

Having determined the amount of preformed creatinine, this may be subtracted from the result just obtained to give the amount of creatine.

Folin's Original Method.—This method is given because it is still preferred by many workers. A solution of potassium bichromate solution serves as a standard.

Creatinine.—Place 10 cc. of urine in a 500 cc. volumetric flask and add 15 cc. of a saturated solution of picric acid and 5 cc. of 10 per cent. solution of sodium hydrate. Shake the mixture thoroughly and allow it to stand for five minutes. Then fill the flask to the 500 cc. mark and after mixing, pour a portion into one of the cups of the colorimeter. Set the other cup, filled with $\frac{N}{2}$ potassium bichromate solution (24.55 gm. to the liter) at the 8 mm. mark. Take the average of several readings. 8.1 divided by the figure obtained multiplied by the total volume of urine gives the daily output of creatinine.

Creatine (Folin-Benedict and Myers' method).—Pipette two 10 cc. portions of urine into two 100 cc. Erlenmeyer flasks, and add to each flask 10 cc. of normal hydrochloric acid. Heat the flasks in the autoclave at 20 pounds pressure for thirty minutes to convert creatine to creatinine. Then use the contents for creatinine determinations as given above, adding to each flask 10 cc. of 10 per cent. sodium hydrate using 10 cc. instead of 5 cc. to overcome the increased acidity. The result represents creatine and creatinine. Creatine may be obtained by subtracting the amount of preformed creatinine.

Normal Quantity.—The normal output of creatinine nitrogen on a standard diet is from 0.50 to 0.66 gm. per day. The creatinine nitrogen makes up from 3.2 to 4.5 per cent. of the total nitrogen.

NITROGEN PARTITION.

The principal non-protein nitrogenous constituents of the urine are urea, ammonia, creatinine, and uric acid, which ordinarily compose about 95 per cent. of the urinary nitrogen. The remainder is composed of small amounts of creatine, purine bases, pigments, amino-acids, hippuric acid, etc. Ordinarily the factors considered as of clinical importance are total nitrogen, urea nitrogen, and ammonia nitrogen. The amount of nitrogen excreted in various forms is determined, and the total nitrogen is ascertained. With these figures, percentages may be computed. Suppose, for example, the total nitrogen be 9.7 gm., the urea nitrogen 8.4 gm., the ammonia nitrogen 0.26 gm., and the creatinine nitrogen 0.64 gm. Then the distribution would be as follows:

Urea nitrogen = 8.40 ÷ 9.7 = 86.6 per cent.
Ammonia nitrogen = 0.26 ÷ 9.7 = 2.7 "
Creatinine nitrogen = 0.64 ÷ 9.7 = 6.6 "

The amounts of all these substances should be reduced to terms of nitrogen before the computations are made.

CHEMICAL COMPOSITION OF NORMAL URINE.

Folin has made practically complete examinations of thirty urines obtained from six individuals who were fed upon a so-called standard diet which contained the amount of protein demanded by Voit for individuals of their body weight. The diet contained about 119 gm. of protein and approximately 148 gm. of fat and 225 gm. of carbohydrates. The average weight of the individuals was 63.4 kilos., ranging from 56.6 to 70.9 kilos. The results were as follows:

	Final average, gm.	Minimum, gm.	Maximum, gm.
Ammonia nitrogen	0.70	0.55	0.85
Total nitrogen	16.00	14.80	18.20
Urea nitrogen	13.90	12.80	16.20
Creatinine nitrogen	0.58	0.50	0.66
Uric acid nitrogen	0.12	0.08	0.15
Undetermined nitrogen	0.60	0.41	0.85
Total sulphur as SO_3	3.31	3.11	3.73
Inorganic SO_3	2.92	2.67	3.25
Ethereal SO_3	0.22	0.19	0.25
Neutral SO_3	0.17	0.13	0.19
Total phosphates as P_2O_5	3.87	3.44	4.50
Chlorine	6.10	5.60	6.90
Volume of urine, twenty-four-hour amount	1430 cc.	1196 cc.	1812 cc.

The nitrogenous constituents may be expressed in terms of percentage with regard to the total nitrogen content.

	Final average per cent.	Minimum per cent.	Maximum per cent.
Urea	87.50	86.20	89.40
Ammonia	4.30	3.30	5.00
Creatinine	3.60	3.20	4.50
Uric acid	0.80	0.60	1.00
Undetermined	3.75	2.70	5.30

DETECTION OF MERCURY IN THE URINE.

The exact diagnosis of mercury poisoning is of importance on account of the slow development of symptoms and the efficiency of modern treatment when instituted early, as has been shown by the work of Lambert and Patterson.

Reagents Required.—(1) Hydrochloric acid, C. P. (2) potassium chlorate dry. (3) copper wire, 18 gauge (Brown and Sharpe wire gauge). (4) dentist's gold foil; J. M. Ney's gold velvet cylinder, cohesive, No. $\frac{1}{4}$ is suitable for this purpose.

Procedure.—(Method of Vogel and Lee.) About 150 cc. of the urine are placed in a beaker, acidulated with about 5 cc. of HCl (C. P.), and boiled over a free flame until the bulk has been reduced to about 25 to 30 cc., when 2 to 3 cc. HCl are added to restore the loss by evaporation. Then sufficient dry potassium chlorate is stirred in to render the mixture practically colorless, and about 30 cc. of distilled water are added. The mixture is boiled again over the free flame to drive off the chlorine until its odor cannot be detected. A piece of copper wire, about 4 cm. long, is bent twice upon itself and is cleaned by boiling for a few moments in a test-tube with a little dilute HCl which may be poured off and replaced with two or three changes of distilled water. The wire is then dropped from the test-tube directly into the beaker of evaporated urine. It is allowed to remain here for two or three hours, or if no deposit be apparent on the wire, for a longer period of time, when it is transferred with a glass rod and carefully washed with a little distilled water. After gentle drying with filter paper, it is slipped into a piece of glass tubing (about 3 or 4 mm. diameter and

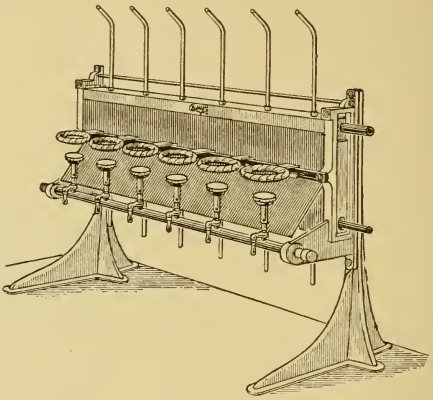


FIG. 72.—Kjeldahl's distilling apparatus for a number of specimens.

15 cm. long) with one end sealed. The wire should not be touched with the fingers. It is then pushed down to the closed end of the tube. After it is inserted a small piece of dentist's gold foil, to occupy a position about 2 cm. above the upper end of the bent copper wire. The tube is held in a horizontal position and the end holding the wire is heated intermittently and gently over a low flame, preferably that of a spirit lamp (Fig. 73). Only the wire and not the foil should be heated. The gold foil should be watched carefully during this process for any appearance of silvering. When only a small quantity of mercury is present, there may be merely tiny silvery spots visible only with a magnifying glass, while with larger quantities there will be a readily perceptible amalgam. After the test has been completed, the open end of the tube may be sealed and the tube kept as a permanent record.

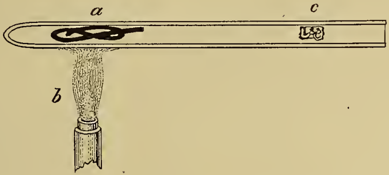


Fig. 73.—Vogel and Lee's test for mercury. *a*, copper wire; *b*, flame of alcohol lamp; *c*, gold foil in capillary glass tube.

Vogel and Lee state that the test is given with a dilution of mercuric chloride as low as 1 to 1,000,000, containing 0.00002 gm. of mercury. In stomach contents and urine, it was found that 1 mg. in 100 cc., equaling a dilution of 1 : 100,000, could be detected by allowing the copper wire to remain in the fluid two hours. Before attaching diagnostic significance to the results, inquiry should be made as to taking of calomel, since it has been found that a positive test will be secured after ingestion of only 2 grains.

TESTS FOR THE DETERMINATION OF RENAL FUNCTION.

It is well recognized that there is by no means an invariable relationship between the evidences of renal tissue damage such as the quantity of albumin and the number and type of tube casts on the one hand and the excretory capacity of the kidneys on the other. Furthermore, it is evident that a determination of the constituents of the urine with the object of detecting an accumulation of waste products by the body can have a narrow range of usefulness unless there is a definite knowledge both of the intake of food materials and the determination of excretion by other channels such as the feces, perspiration, and respiration. Complete metabolic studies of this type are manifestly out of the question in ordinary clinical work, and are not always readily accomplished for clinical purposes even in well equipped hospitals.

The introduction by Folin of colorimetric methods for blood analysis has placed at the disposal of the clinician a means of studying the chemical composition of the blood and of ascertaining whether there be an accumulation of waste products. The information so derived, correlated with the bedside findings and properly interpreted, is of great assistance in evaluating the functional power of the kidneys.

Of methods which have been offered as tests for the functional power of the kidneys, two stand out as of the greatest clinical value. The first is the determination of the ability of the kidney to excrete phenolsulphonephthalein. Numerous tests employing as their basic principle the excretion of dye substances have been proposed, but phenolsulphonephthalein seems best adapted to practical usage. The second is the so-called "test-meal for renal function" proposed by Mosenthal, who adopted the suggestion of earlier workers that the response of the kidney to a full meal containing a reasonable amount of fluids, purins, and salts, varied in health and disease, and formulated a method whereby this might be studied by having the urine voided at fixed intervals after such a meal.

As Macleod remarks, while the so-called renal tests are not free from criticism, they have contributed very useful information; the excretion by the kidney of a chronic nephritic of a urine of more or less fixed specific gravity would suggest an impairment of the resorbing mechanism, and the failure of a kidney to excrete the proper amount of dye suggests an impairment in the filtering apparatus, but hard and fast rules cannot be applied and probably the tests must be interpreted at present for the kidney as a whole.

Excretion of Phenolsulphonephthalein as a Test for Renal Function.—(Method of Rowntree and Geraghty.)

Apparatus and Reagents Required.—(1) Hypodermic syringe, all glass, accurately graduated and calibrated, preferably one of Becton-Dickenson manufacture, the type referred to as "Tuberculin," with a long slender barrel. (2) Ampoules each containing 1 cc. of sterile phenolsulphonephthalein sterile solution, representing 6 mg. of the dye. These are marketed by Hynson, Westcott and Dunning, of Baltimore, Md. (3) Standard dilution made by diluting 6 mg. of the phenolsulphonephthalein with a little water, adding 10 cc. of 25 per cent. sodium hydrate, and bringing the volume to 1000 cc. with distilled water. If not exposed to strong light this keeps for a period of months. (4) Solution of sodium hydrate (10 to 25 per cent. may be used, usually 15 per cent). (5) One thousand cc. graduate or volumetric flask. (6) Colorimeter. The colorimeter used for other colorimetric work may be employed, either the Duboscq, Kober, Bock-Benedict, or Myer. The Autenrieth-Koenigsberger colorimeter made by Hellige of Freiberg was modified for this work and has been very generally employed (Fig. 74). It has the advantage that the percentage may be read directly from the scale. Hynson, Westcott and Dunning have put out a small inexpensive colorimeter, which is simple though not accurate.

Procedure.—The patient is given a glass of water to drink to ensure free urinary secretion. One-half hour later the bladder is emptied, by catheterization if necessary. A sterile hypodermic syringe is filled with exactly 1 cc. of the phenolsulphonephthalein solution, which contains 0.006 gm. of the dye. This is injected preferably into the lumbar mascular tissue. The area selected should be free from edema. The bladder is emptied one hour after the injection of phenolsulphonephthalein and again at the end of two hours either by voiding or by catheterization. The two specimens so obtained are examined separately. Enough sodium hydrate solution is added to alkalinize, so that the color will develop fully, and the total volume in each specimen is brought up to 1000 cc. with clear water. If the color be faint, it is well to bring the volume up to only 500 cc. The cup of the colorimeter is filled with some of the resulting mixture and the reading taken. When a Hellige colorimeter is employed, the percentage may be read off on the scale directly. If the urine were diluted to 500 cc. only, the reading should be divided by 2.

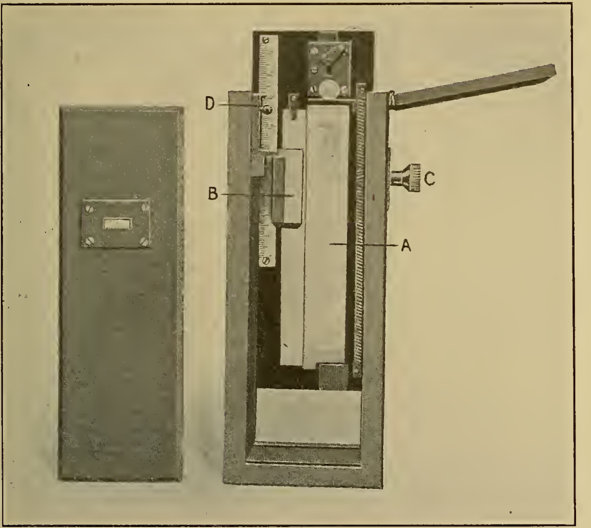


Fig. 74.—Hellige's colorimeter. (Simon.)

When it is desired to employ the Duboscq or a similar colorimeter dilute a small quantity of the standard solution already described (6 mg. of phenolsulphonephthalein in 1 liter of water) with an equal quantity of distilled water. The left hand cup of the colorimeter should be half-filled with this, and then adjusted to a depth of 10 mm. The urine, alkalinized and diluted as directed, is placed in the right-hand cup, which is then moved up and down until the two sides of the visual field are the same, when the reading is taken. The calculation is made by this formula; $\frac{500}{R}$ = per cent. phenolsulphonephthalein excreted. For example, with the standard set at 10, if the reading were 20, the percentage of dye excreted would be $\frac{500}{20} = 25$.

The sum of the reading obtained from the two specimens represents the percentage of the dye excreted in two hours.

Estimation of the Function of the Separate Kidneys.—To accomplish this purpose, catheterization of the ureters is necessary. The patient is given 200 to 400 cc. of water to drink about a half hour before the test to ensure the free flow of urine. For the technic of ureteral catheterization, the reader is referred to standard texts on genito-urinary surgery. After the catheters have been introduced, Rowntree and Geraghty direct that the cystoscope be withdrawn, placing around the free extremity of one of the catheters a bit of adhesive to distinguish the right from the left. It is well to be certain that there is a free flow of urine from the catheters before injecting the drug, in order to avoid the possibility of obscuring the results should there be inhibition of the urinary flow due to ureteral catheterization. In these cases, the drug is usually given intravenously rather than intramuscularly, on account of quicker elimination. As it is the relative amount of work each kidney is capable of doing that is being studied, it is sufficient to collect the urine from the ureter catheters for a period of twenty minutes after the appearance of the drug on one side. Provided neither ureter catheter becomes plugged during this twenty-minute collection period just as accurate a comparative estimate of the secretory power of the two sides can be made as if the ureter catheters were allowed to remain in position for a longer time. The ends of the catheters are placed in test-tubes so that the urine may be collected. Into each test-tube are placed a few drops of 15 per cent. sodium hydrate. The tubes are watched for the first appearance of the red color and the interval of time since injection is noted.

Normal Results.—In normal cases the drug appears in the urine after intramuscular injection in from five to ten minutes and in from three to five minutes after intravenous administration. Forty to 60 per cent. of the amount given is recovered at the end of the first hour and from 15 to 25 per cent. at the end of the second hour, making a total recovery for the two hours of 61 to 85 per cent. The excretion is markedly lower in all forms of nephritis, and is apparently more or less proportional to the amount of involvement of renal tissues. In chronic passive congestion, it is low, but returns rapidly to normal with improvement in circulation, while it fails to do so when the passive congestion is accompanied by nephritis. In surgical kidney conditions where the ureters can be catheterized and the amount of drug excreted by each kidney compared, the test often gives extremely valuable information. In a kidney which is the seat of hypernephroma, advanced tuberculosis, or calculus pyonephrosis, the time of appearance of the drug will be greatly delayed, and the amount may vary from a mere trace to a very small amount, while from the other side, a normal time of appearance and an amount of drug nearly equal to the excretory power of two kidneys will point to satisfactory compensation by the normal kidney.

With urinary obstruction from hypertrophy of the prostate or stricture, and resulting back pressure on the kidneys, the time of appearance is delayed, and the amount of drug excretion diminished. With the relief of this back pressure, the recuperative power of the kidneys may be gauged by the degree of increase of elimination of the drug in a series of tests, over a period of days or weeks. The test is of value in diagnosing uremia from conditions which simulate it, and by very low readings, in indicating the fact that uremia was impending even when clinical evidence is absent.

With this, as with all other functional tests, too much reliance cannot be placed on the findings in any single test or series of tests. Though strikingly accurate information is secured by its use in many cases, in others, the results do not check with other clinical findings and are difficult to explain. For this reason the data secured by the use of the test should be correlated with all other available clinical evidence before coming to a decision in any case.

Excretion of Indigo-carmin.—The use of indigo-carmin in chromoureteroscopy has been advocated by Thomas. The onset, character, and intensity of coloration of the dye as it is ejected from the urethral orifices after intramuscular injection is observed cystoscopically. After introducing an evacuation-irrigation cystoscope, under local or general anesthesia, 20 cc. of 0.4 per cent. solution of indigo-carmin at blood heat are injected deep into the gluteal muscles. The bladder is drained, washed thoroughly, and filled with sterile boracic acid solution (150 cc. in the case of males, 200 cc. in females). Maintaining a brilliant illumination, the bladder wall is studied carefully. Not only the time of appearance of the dye but the intensity of the color should be noted. The appearance of jets of blue urine from the urethral orifices renders urethral catheterization easy in many instances where it might be extremely difficult to see the openings. From normal kidneys, indigo-carmin is eliminated as dark blue in from four and a half to twenty minutes; as light blue not later than fifteen minutes. Renal function is seriously impaired when the color does not appear in fifteen to twenty minutes.

Thomas feels that this test is applicable in surgical affections of the kidneys and ureters, especially in hydronephrosis, pyonephrosis, pyelonephritis, and chronic tuberculosis involving approximately one-third of the parenchyma; but is of no value in movable kidney, in the absence of urethral kink or hydronephrosis, pyelitis, early tuberculosis, most forms of nephritis, and small tumors not involving the greater part of the parenchyma. Thomas has described an "index of elimination," determined by dividing the quantity eliminated during the first hour by the quantity eliminated during the third hour after injection. The index for a series of normal individuals was 5.1. He thinks that when the amount excreted during the third hour equals or exceeds that eliminated during the first hour, serious operative intervention, namely prostatectomy, is contra-indicated, unless the total amount eliminated in three hours exceeds 20 per cent.

For the determination of quantity, the urine is collected by catheterization or voluntary micturition, and placed in 1000 cc. volumetric flasks, reserving a flask for each of three periods of one hour each. Clear water is added to each specimen to bring the total volume to 1000 cc., and portions are compared in the colorimeter with a standard prepared by placing 20 cc. of 0.4 per cent. solution of the dye in a volumetric flask and adding distilled water to 1000 cc. When used in the Hellige colorimeter, the percentage is read directly on the scale. For the Duboscq or similar instruments, a portion of the standard is diluted with three times its volume of distilled water and used in the left hand cup of the colorimeter which is set at 10 mm., while the diluted urine is placed in the right hand cup, comparing in the usual way, and calculating with the following formula:

$$\frac{250}{R} = \text{per cent. indigo-carmin excreted.}$$

Mosenthal Test-meal for Renal Function.—The patient is directed to take a full meal of an ordinary dietary, with the usual amount of meat, but to take neither fluid nor food between meals. The prohibition against taking fluid between meals should be emphasized. During the day when the patient is taking this diet, the urine should be collected at two-hour intervals, the bladder being emptied each time as completely as possible. The specimens are kept in separate bottles, properly labelled with the time at which the contained urine was voided. A time schedule should be given to the patient. For example, he should be directed to collect the specimens at 8 and 10 A.M., and 12 N., and at 2, 4 and 6 P.M., and again three hours after the evening meal. Any urine passed after this time during the night should be kept and mixed with the specimen voided in the morning, which would represent the output of ten or twelve hours.

Mosenthal states that under these circumstances the normal response would show a maximum specific gravity of 1.018 or more. The specific gravity should vary at least 9 points between the highest and lowest readings. The night urine should be small in amount, that is, less than 400 cc., and of high specific gravity, 1.018 or over.

Signs of a diminished renal function are, (1) a lowering of the maximal specific gravity; (2), a fixation of the specific gravity; and (3) a nocturnal polyuria.

Mosenthal has proposed a table giving various degrees of impairment as indicated by the results of the test-meal when compared with the results of other tests.

Degree of impairment of renal function.	Night urine.		Variations in specific gravity when the highest specific gravity is			
	Cc.	Sp. gr.	1.018	1.017 –15	1.014 –13	1.012 and less
Normal . . .	400 –	1.018 +	9 +			
Slight . . .	401–600	1.016-17	8.5	6 +		
Moderate . .	601 – +	1.015 –	4 –	5 and 4	6 +	
Marked . .	. .	. .	. .	3 –	4 and 5	6 +
Maximal . .	. .	. .	. .	. .	3 –	5 –

The salt and nitrogen content of the various specimens may be determined also, but Mosenthal states (and most workers agree with him) that the simple procedures of measuring the volume of the urine and of determining the specific gravity yields sufficient data to give an adequate idea of the renal function in many respects. The Mosenthal diet test is easily employed and is of especial value in early cases of nephritis, for it gives positive indications and reaches the highest degree of impairment before other tests.

It is to be borne in mind that so-called "dissociation of renal function" is seen in some of the anemias. There is a marked fixation of the specific gravity at a low level and a nocturnal polyuria, while the amount of blood urea, the phenolsulphonephthalein test, and Ambard's coefficient show findings little difference from normal.

Ambard's and McLean's Coefficients.—Ambard claims to have shown that the elimination of at least certain substances is carried on by the kidneys according to definite laws which are capable of mathematical expression. These laws he reduced to a mathematical formula known as Ambard's coefficient. The relative constancy of this as regards the excretion of urea was claimed by Ambard.

The formula has been modified by McLean so that the index may express directly the changes in the rate of excretion. One hundred represents the normal finding and corresponds with a value of 0.08 for Ambard's coefficient. In this country, the McLean index has come into wider use than the Ambard coefficient.

McLean's index is expressed as follows:

$$\text{Index} = \frac{(\text{gm. urea per twenty-four hrs.})\ \sqrt{\text{gm. urea per liter of urine}} \times 8.96}{\text{Wt. in kilos.} \times (\text{Gm. urea per liter of blood})^2}$$

In order to secure free excretion of urine, the patient should drink 150 to 200 cc. of water. Half an hour later the bladder is emptied completely, by catheter if necessary. The time is noted exactly to a minute. The urine is collected at the end of seventy-two minutes. This time period is selected for the sake of convenience, since it is one-twentieth of twenty-four hours. Should an error of a few minutes be made of the second specimen in the collection of urine, the exact time should be noted and the output for twenty-four hours should be calculated on this basis. The amount of urea is determined in this specimen.

Thirty-six minutes after the beginning of the period, 10 cc. of blood are obtained from the vein of the arm, clotting being prevented as usual by the addition of sodium or potassium oxalate (see page 167) and the amount of urea determined (see page 177).

The patient must take neither food nor fluid during this period. If any urine be voided during the interval, it must be added to that obtained at the end of the period. The urine must be carefully collected and handled with care to avoid any loss, and the total quantity should be measured accurately. The patient's weight should be taken on the day of the test.

The following data is now available: the quantity passed in seventy-two minutes, which may be multiplied by 20 to give the twenty-four-hour amount; the gm. of urea per liter of urine, from which may be determined the amount in twenty-four hours; the grams of urea per liter of blood; and the patient's weight in kilograms.

These figures may be substituted in the formula. The calculation may be greatly simplified by a slide rule adapted to the formulas (Fig. 75). This may be secured from the Keuffel and Esser Co., 127 Fulton Street, New York City.

Example.—McLean gives the following example for calculation of the index by the aid of the slide rule:

Gm. urea excreted per twenty-four hours, $D = 20.0$
Gm. urea per liter of urine, $C = 11.0$
Gm. urea per liter of blood, $Ur = 0.33$
Gm. body weight, in kilos, $Wt = 55.0$

$$\text{Index} = \frac{D\sqrt{C} \times 8.96}{Wt. \times Ur^2} = \frac{20.0\sqrt{11.0} \times 8.96}{55.0 \times (.33)^2}$$

This may be worked out with the slide rule as follows:

1. 55.0 on the weight scale is set opposite 20.0 on the *D* scale; first position.

2. The hair-line on the runner is moved to 11.0 on the *C* scale; second position.

3. The slide (center portion) is moved so that 3.3 on the *Ur* scale is at the hair line on the runner; third position.

4. The reading is now made at the arrow which is pointing toward the scale *I*. In the example (see illustration), it points to 100, and the index is therefore 100.

Interpretation of Results.—McLean has shown that the findings correspond very closely with those of the phenolsulphonephthalein test. While 100 is taken as representing normal, it is stated that usually the index is between 100 and 200, and variations between 80 and 300 are not infrequent in normal persons. He states that an index

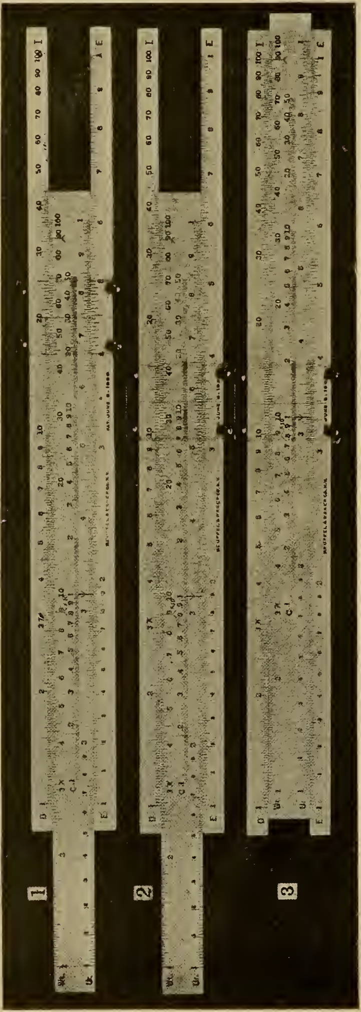


FIG. 75.—Ten-inch slide rule adapted to the calculation of the index of urea excretion. (McLean.)

below 80 is to be considered as abnormal, though not necessarily seriously so. In renal disease an index below 50 is indicative of a considerable degree of renal impairment of functional ability. It is believed by him that the amount of damage to the kidneys is increasingly greater as the index is lower and tends to approach zero. A low index, however, may be only temporary, as in passive congestion of the kidneys due to heart failure or in acute nephritis, and may return to normal by improvement of the underlying condition.

DETERMINATION OF TOLERANCE TO SODIUM BICARBONATE AS A TEST FOR ACID INTOXICATION.

Method of Sellards.[1]—Sodium bicarbonate is given by mouth or intravenously in 5 gm. doses every two or three hours to determine the amount which is needed to make the urine neutral or alkaline to litmus (or to reduce the reaction to that of normal blood, pH 7.4). The bicarbonate should be given in a moderate amount of water and the patient should void before each administration. Specimens of urine which are not distinctly acid to litmus should be boiled. Boiling converts the bicarbonate to carbonate so that the reaction to litmus will be sharper.

Normally 5 to 10 gm. are sufficient to change the reaction, while with acidosis more will be required, even as much as 140 gm. in severe cases. Palmer and Van Slyke have called attention to the possibility of giving unnecessarily large and possibly injurious amounts of bicarbonate, since in most of the pathological cases which they studied the urine did not become neutral until the blood plasma bicarbonate content had reached a much higher figure than normal. They think that the therapeutic use of bicarbonate should be controlled by a determination of the plasma bicarbonate. They found that for each 42 pounds of body weight, it is necessary to give 0.5 gm. of sodium bicarbonate to raise the plasma CO_2 1 volume per cent. The amount given should be sufficient to raise the plasma bicarbonate 71 to 75 volume per cent.

BACTERIOLOGICAL EXAMINATION OF URINE.

For bacteriological examination, the urine should be obtained with great care. A catheter should be passed under aseptic conditions and the urine drawn into a sterile container.

Examination of Urine for Tubercle Bacilli.—The urine should be obtained as described above, as though it were desired for cultural purposes. Fill 2 centrifuge tubes with urine and centrifuge for five minutes. Then pour off the supernatant fluid, add fresh urine, and again centrifugalize. Repeat the operation a number of times, until the sediment represents that obtained from 150 to 200 cc. of urine.

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[1] Principles of Acidosis, Harvard University Press, Cambridge, 1919, p. 107.

If a precipitate of urinary salts be present, the sediment should be "washed" with freshly distilled water by filling the tubes with distilled water and centrifugalizing for five minutes. The supernatant fluid is poured off and the process repeated. The sediment now may be spread upon a slide, adding a drop of Mayer's egg-albumen if necessary to facilitate adherence. After drying and fixing in the usual way, the film is stained by steaming with carbol-fuchsin for two minutes, as directed in the chapter on Sputum. In decolorizing, especial pains must be taken to rule out acid-fast organisms other than the tubercle bacillus, especially the smegma bacillus. The slide should be decolorized and simultaneously counterstained by pouring Pappenheim's rosolic acid decolorizing fluid over the film and draining it off five or six times. Inoculation of two guinea-pigs with the sediment obtained by centrifugalizing the urine is desirable.

For assistance in the identification of other organisms, reference should be made to the chapter on Bacteriological Methods. Those for which search is made commonly include, in addition to the tubercle bacillus, the gonococcus, colon and typhoid bacilli, the pyogenic cocci, and the streptococcus.

THE TWO- AND THREE-GLASS TESTS.

Simple tests sometimes used for localizing the site of an inflammatory condition in the urethral tract are the two- or three-glass tests. In any of these tests the patient should have held his urine for at least four hours previous to making the tests. Conical glasses or tall cylinders with a capacity of about 200 cc. should be employed.

Two-Glass Test.—The urine is voided into the first glass until about 30 cc. are obtained when the flow is stopped. The remainder is allowed to pass into the second cylinder. The first glass is presumed to contain the washings from the urethra; the second glass will receive that from the organs which are proximal to (above) the external sphincter, that is, the posterior urethra, prostate and bladder. However, the test may be misleading, since with posterior urethritis there may be little discharge from the posterior urethra to escape into the bladder so that the second specimen may be clear, giving rise to the false impression that the anterior urethra is the seat of the trouble. Then with a simple anterior urethritis, there may be little urine to void so that the anterior urethra is imperfectly cleansed and the second glass is cloudy, when with a greater quantity of urine, it would be clear.

Three-Glass Test.—This test is similar to that just described, except that the urine is passed in a continuous stream into three glasses, into the first two as it is in the two-glass test, and into the last with as much straining as possible, so as to express secretion from the prostatic ducts. It is subject to the same fallacies as the two-glass test.

Four-Glass Test.—A more accurate estimate of the source of inflammatory products in the lower urinary tract may be secured by the *four-glass test.* To carry this out, the patient must report with a moderately well filled bladder. The test is done as follows:

Glass 1. Anterior Urethral Washings.—Before the patient voids any urine, the anterior urethra is gently flushed out with sterile water by means of a hand syringe or irrigator. These washings are collected in the first glass, and any pus or shreds will be obviously of anterior urethral origin.

Glass 2. Post-urethro-vesical.—The patient now voids into the second glass what he considers to be a third of his bladder urine. Pus shred or blood contained in this glass must originate from the posterior urethra or bladder.

Glass 3. Vesical.—The patient voids into a third glass a further portion of his urine which may fairly be held to represent unmixed bladder urine.

Glass 4. Prostato-vesicular.—The patient's prostate and vesicles are now massaged, after which he voids the remainder of his urine into a fourth glass. This glass should contain abnormal elements from the prostate and vesicles if they are diseased.

Examination of Prostatic Fluid.—In infection of the prostate or when there is a history of previous gonorrheal infection it is always desirable to examine the prostatic fluid. This is obtained by prostatic massage. The patient is directed to bend over, leaning against a table for support. The operator inserts the index finger, well greased, and covered with a rubber cot into the rectum and strokes the prostate from above downward with gentle massage first on one lobe and then on the other, until secretion appears. This is caught as it drops from the meatus upon a slide. In case no strippings are secured at the meatus, the urine should be voided immediately and the sediment examined.

URINARY FINDINGS IN MORBID CONDITIONS.

The diagnosis of renal diseases should not be made from the results of urinary examination alone. In a consideration of the clinical evidence, the urinary findings, no matter how complete, furnish only part of the evidence. Other features which should be given full consideration are the patient's history, the condition of the heart and the bloodvessels, the blood-pressure, the presence and absence of edema, and the presence of signs of uremia. The results of determinations of the amount of urea, creatinine, and uric acid and the CO_2 combining power of the plasma, furnish data of great value in the study of nephritis. The significance of the findings have been referred to in the section on Blood Chemistry.

The classification of renal disorders which is used here is unsatisfactory both to pathologists and clinicians. It is utilized partly because it is followed in most of the standard works on medicine, but chiefly because no better one has been offered for clinical purposes.

Acute Nephritis.—The quantity of urine is greatly reduced, even to a daily output of 100 to 150 cc. The color is dark, usually smoky, rarely a bright red. The specific gravity is high, usually over 1.025. The sediment shows blood corpuscles in various stages of hemolysis, epithelial cells, and casts. The casts include those of the hyaline and granular variety, and those containing red cells, epithelium, leukocytes. Albumin is present in large amounts. The phenolsulphonephthalein excretion is reduced. Should recovery ensue, there is a gradual improvement in all these signs. The quantity of urine increases, the albumin diminishes in amount; the red cells disappear while casts often persist for a longer period of time. A definite proportion of cases which make a partial recovery gradually develop chronic nephritis, and the urinary findings are those which are expected in this condition.

Chronic Parenchymatous Nephritis.—The specific gravity is usually increased in proportion to the diminution in the amount of urine. The color is yellow, rarely smoky. Albumin is present in rather large quantities. Microscopically, the sediment is seen to include occasional red cells, an increased number of leukocytes, hyaline, granular, epithelial casts, and occasionally fatty and red blood cell casts. There are also epithelial cells from the kidney and the pelvis. Phenolsulphonephthalein excretion is impaired. The blood often does not show as great retention of nitrogenous substances as is seen in chronic interstitial nephritis, but may show a decided increase in the amount of chlorides.

Chronic Interstitial Nephritis.—This includes the secondarily contracted kidney (small white kidney) and the primary granular kidney. From the standpoint of urinary findings, there is no way of differentiating between these types. Usually the urine is much increased in amount. Nocturnal urination may be the first symptoms of which the patient complains. The color is light yellow and the specific gravity is persistently low, ranging from 1.005 to 1.012. Albumin may be found in a small quantity but is often absent, and it may be necessary to examine a number of specimens to detect any. Casts, which are usually of the hyaline variety, with possibly a few of the granular type, are usually in a rather small number and may be absent at times. In the arteriosclerotic form of contracted kidney, the quantity is normal or reduced, the specific gravity is normal or high, there are hyaline and granular casts, and albumin is present, varying somewhat in amount according to the diet and exercise. The phenolphthalein excretion diminishes as the pathological process advances. Urine obtained after a Mosenthal test diet shows fixation of the specific gravity and an increase in the nocturnal quantity. The blood shows an increase in the amount of uric acid in many incipient cases; an increase in uric acid and urea in moderately advanced cases; and increases in these two substances and creatinine as well in cases of a still more severe type.

Amyloid Kidney.—In this rather uncommon condition the urine is increased in amount, pale and clear, of low specific gravity, and usually shows an abundant amount of albumin. Hyaline, granular, and fatty casts, occasionally those of amyloid type, are found.

Pyelitis.—The urine is turbid, due to the presence of pus. In addition to pus, there may be large amounts of mucus and desquamated epithelium. Blood is occasionally present, giving the urine a smoky appearance. Albumin is present in amounts ranging from a trace to a measurable quantity. The pus often varies in amount. It may be intermittent when only one kidney is involved and the ureter is temporarily blocked. At times, casts of the kidney tubules or moulds of the calyces may be passed. The reaction of the urine varies according to the type of the infecting organism. With a B. coli communis infection, it is usually highly acid. Microscopically, one finds leukocytes in various stages of preservation, depending upon the reaction. The outline is usually well preserved in acid urine, while in alkaline urine, the white cells are often partially dissolved, so much so that definite morphology may be almost distinguishable. In addition epithelial cells are seen, not infrequently those of the caudate type predominating. Red blood cells are seen at times. Since pyelitis is almost always accompanied by an involvement of the kidney substance itself, casts are to be expected. They may be hyaline, but it is not unusual to find them containing leukocytes, epithelial cells and red blood cells. Microörganisms are present, the varieties more frequently responsible for pyelitis including the tubercle bacillus, bacilli of the colon group, B. proteus vulgaris, and the staphylococci.

Nephrolithiasis.—All cases of renal stone when complicated by infection are accompanied by varying degrees of pyelonephritis so that pus may be seen in the urine over a long period of time. Indeed, the diagnosis of pyelitis is not complete until *x*-ray examination has been made to exclude the possibility of calculus. The urine, which in the absence of complicating pyelitis may have been quite clear, will often show at the time of colic red blood cells ranging in number from a very few detected only microscopically, to a quantity sufficient to render the urine turbid.

Passive Congestion.—The kidneys are frequently involved in other conditions, to which reference has been made in discussing the causes of albuminuria and hematuria. The passive congestion occurring in failure of cardiac compensation is of especial importance from the standpoint of differential diagnosis.

The amount of urine is small, at times almost to the point of anuria. It is dark in color, often smoky or of reddish tinge, due either to the high concentration or the admixture of blood. The specific gravity is high, and the reaction usually acid in freshly voided specimens. Albumin may be present in large amount. The sediment, which may be considerable, shows casts of the hyaline and granular varieties, not infrequently those of epithelial and red blood cell types. Red blood cells and renal epithelial cells may be seen. The excretion of phenolsulphonephthalein is low but increases as cardiac compensation is restored.

Cystitis.—Urination is usually frequent and painful. The urine is turbid. Alkaline decomposition in freshly voided specimens is frequent in chronic cases, the sediment showing amorphous and triple phosphates. In acute or chronic B. coli infections, the reaction is almost invariably acid. Pus may be present in macroscopic amounts. Mucus is seen at times, occasionally enough to form a ropy deposit. Albumin is present. Microscopically, red cells, epithelial cells (chiefly of the large round variety), leukocytes, and bacteria are found. Casts are not seen unless there be an involvement of the kidneys also.

Urethritis.—The urine contains pus in varying quantities. Occasionally red blood cells may be seen in the acute stage, and albumin is often present when the inflammatory reaction is severe. The two-, three-, or four-glass test may be of assistance in localizing the exact seat of the process.

Diabetes Insipidus.—The characteristic finding in this condition is a large quantity of pale urine of low specific gravity (1.001 to 1.005) containing no albumin or sugar.

Diabetes Mellitus.—The amount of urine is large, ranging from 3 even to 20 liters. It is generally of light color and of highly acid reaction. Albumin is not infrequently present. Sugar is present in the form of dextrose, the amount ranging from a fraction of a per cent. to 10 per cent. (the latter figure representing a maximum which is seldom reached). The percentage is of course of secondary importance, the total amount per diem being of greater significance. This may range from 300 to 640 gm., and Osler states that there are exceptional cases with a daily output of from 1 to 2 pounds. In the more severe cases, acetone, diacetic acid, and β-hydroxybutyric acid are present and the amount of ammonia is high. Chemical examination of the blood is of importance, and the laboratory procedures should include a determination of the amount of blood sugar and of the CO_2 combining-power of the blood plasma.

URINALYSIS WITH RELATION TO LIFE INSURANCE.

To life insurance companies, the results of urine examinations assume great importance. The examiner should perform this part of his examination with the same care which he bestows upon the rest of the work. In taking the history, especial attention should be paid to the frequency of urination, nocturnal urination, and to pain on urination. The urine should be passed in the presence of the examiner. In the case of women, it should be passed in an adjoining room, and the examiner should be morally certain that it had been passed by the applicant. He can reassure himself to a certain extent by determining that the specimen is warm. Specimens should not be examined which are brought or sent in by the applicant.

SYNOPTIC TABLE OF FINDINGS IN CERTAIN MORBID CONDITIONS.

Condition.	Daily amount.	Color.	Specific gravity.	Amount of albumin.	Sediment.	Phenolsulphonephthalein.	N-protein in blood.	Chlorides in blood.
Acute nephritis . .	Small; as low as 150 cc.	Dark; smoky	High	Large	Red blood cells, white blood cells, hyaline, granular red blood cells, and epithelial casts	Reduced; may be very low	May be increased	Variable.
Chronic parenchymatous nephritis . .	Diminished; as low as 500 cc.	Yellow; rarely smoky	High	Large	Hyaline, granular, fatty, epithelial casts, and occasional red blood cells	Reduced	May not be increased	Often increased.
Chronic interstitial nephritis.	Increased	Light	Low	Small or absent	Occasional hyaline and granular cast	Reduced	Increased in later cases	Normal.
Passive congestion .	Small	Dark	High	May be large	Hyaline and granular casts, red blood cells, epithelial cells	Slightly reduced or normal	May not be increased	Usually normal.
Pyelitis	. . .	Turbid occasionally smoky	. . .	Variable traces	White blood cells, occasional red blood cells, hyaline, granular, leukocyte, epithelial and red blood cell casts; bacteria.			
Cystitis	. . .	Turbid	. . .	Trace	White blood cells, occasional red blood cells, bacteria, no casts, bladder epithelium, mucus.			
Urethritis	. . .	Turbid	. . .	None or trace	White blood cells, occasional red blood cells; in acute stage, epithelium, bacteria.			

The routine examination required by most companies includes the gross appearance, the determination of specific gravity, the reaction to litmus, the detection of albumin and sugar. The clarity or turbidity should be noted. The cause of turbidity should be explained on the blank, for the company will desire to know whether turbidity be due to the presence of bacteria or pus, or merely to a precipitation of urates or phosphates. Instructions for differentiating have been given in foregoing paragraphs (see page 218). The specific gravity is of importance chiefly to obviate the possibility of examining a dilute specimen, in which it is more difficult to detect a small quantity of albumin. The limits ordinarily set are between 1.010 and 1.130. The reaction is of slight importance except that alkalinity often indicates decomposition.

Albumin should be tested for first by the heat and acetic acid test. Should this prove negative, the result may be taken as final, but if it be positive, Heller's test should be done. The result of both examinations should be noted, and should the examiner think that they indicate the presence of nucleoprotein, he should make a notation to this effect on the blank. The majority of the companies do not desire the use of more delicate tests than those mentioned, because the significance of faint traces found with these methods is not definitely determined. The companies generally prefer to have the examination for sugar carried out with one of the reduction tests. There is still some preference for Fehling's solution. The solutions should be kept in separate bottles and should be mixed on the day when the test is made as directed in foregoing paragraphs (see page 223). The urine should be fresh to prevent possible loss of sugar by decomposition. When a positive reduction is obtained, the presence of sugar cannot be taken as definitely established until the result has been checked by some method other than one employing the reduction principle. The fermentation test is simple and reliable.

A determination of the amount of urea is sometimes required. It is practically impossible to obtain a twenty-four-hour specimen for insurance purposes, and it is difficult to see what useful purpose is served by ascertaining the amount of urea in a single specimen except to demonstrate that the specimen is sufficiently concentrated to correspond to the specific gravity and that there is an approximate correspondence between specific gravity and urea concentration. The quantitative determination of other bodies, such as chlorides, phosphates, sulphates, and uric acid, can have no possible significance when performed on a single specimen without regard to the diet.

In making the report all abnormalities should be noted upon the blank, but they should not be reported to the home office until a second specimen has been obtained. Should the same abnormality be found again, the fact should be noted with the date of the second examination, and the blank sent to the company. If the second specimen should prove normal, this fact should be noted and the blank should be sent to the home office, which usually requires that a third specimen be obtained and sent to them by mail for examination. When either albumin or sugar has been found once, even though later specimens are clear, the applicant is required usually to sign a statement that his diet has not been restricted during the preceding six months for glycosuria or albuminuria (as the case may be), in order to protect the company against the possibility of accepting a poor risk who has been placed by medical care in favorable condition for examination.

Microscopic examinations are made usually only at the home office of the company or by certain delegated referees or chemists at the larger centers. When sending a specimen for microscopic examination, the examiner should take pains to prevent decomposition by prompt mailing and by the addition of a suitable preservative. Both container and preservative are usually furnished by the company.

When the microscopic examination is to be performed away from the home office, it is desirable that the sediment from at least 15 cc. of urine be obtained by the use of a high speed centrifuge. As much of the sediment as possible should be placed upon the slide. One company has prescribed the following requirements which must be met before doubtful bodies are classed as casts: (1) Both ends should be rounded. (2) Neither end should show tapering, tendency to clubbing, or tails. (3) The sides should be parallel, and the body should show no cracks or indentations. (4) The matrix should be homogenous, except, of course, in the case of granular or cellular casts. (5) At least three perfect specimens should be found before the specimen is reported as positive.

Opinions differ about the acceptability of applicants whose urine shows few hyaline casts, some companies accepting risks where the number is limited to a few in a given slide. This is done because it is recognized that hyaline casts do not always mean kidney disease, for they may result from indiscretions in diet or from excessive exercise. Granular casts, however, usually mean permanent kidney damage and almost invariably are taken as a cause for rejection. The attitude regarding the presence of casts has been summed up by a writer on this subject as follows: "The presence of a few hyaline casts should be treated in the same way as albuminuria. If they persist, the application should be postponed until the inspection of several specimens at some future time has demonstrated the fact that hyalines are no longer present. As long as they are present in the urine there must be some uncertainty as to whether the process through which they are formed is serious or inconsequential, and this opinion holds even though the condition of the applicant is otherwise satisfactory." As the same writer remarks, the life insurance point of view and that of the practitioner is quite different, since when a person applies for life insurance, the examination is necessarily limited to a short time and there is little opportunity for continued observation.

CHAPTER III.

EXAMINATION OF GASTRIC AND DUODENAL CONTENTS.

GASTRIC CONTENTS.

Relative Simplicity of Examination of Gastric Contents.—The examination of gastric contents may be reduced by the practitioner to very simple terms. The more useful procedures can be carried out with a limited equipment and with the expenditure of a relatively small amount of time.

It is highly important to select the proper meal to be given for the purpose in mind, to give more than one type of meal if the circumstances demand, and, in many cases, to repeat the gastric analysis before drawing final conclusions. If, for example, the presence or absence of lactic acid might have considerable influence in making the diagnosis, the meal should be one which contains no lactic acid.

Finally, the results must be interpreted in the light of the clinical findings, and should never be viewed as self-sufficient facts. As has been remarked by Dock, "the possibilities are that in stomach diagnosis, as in other lines, the greatest assistance will come from the patient efforts to appreciate the alterations of function and structure primarily, and by allowing our diagnosis to grow out of these findings, assisted by the other clinical features of the case. . . . Examinations of gastric contents give information that cannot be obtained in any other way, and, though often negative, cannot be excluded without risk of serious error. Such examinations are indicated in all stomach syndromes, in all diseases seriously affecting metabolism and nutrition, and in all diseases that affect the function of the stomach. . . ."

Apparatus required.—The apparatus required is included in the list to be found in the Appendix for "Minimum Equipment for the Clinical Laboratory."

THE GASTRIC JUICE.

General Considerations.—A brief review of gastric physiology is necessary in order to present the considerations underlying the examination of stomach contents for diagnostic purposes. The first stimulus to gastric secretion is a psychic one, and depends upon the gratification of appetite and the pleasure in eating pleasing food. The stimulus caused by psychic influence suffices to cause an adequate flow of gastric juice for a limited period of time. This may last for only fifteen or twenty minutes. Further secretion is excited by the products of protein digestion which act upon the mucous membrane of the stomach and produce a hormone, called gastrin, which when carried by the blood to the cells of the gastric glands, excites them to activity. Furthermore, it is known that fat has a distinctly inhibitory effect upon the secretion of gastric juice, and that the presence of oil in the stomach materially diminishes the amount of secretion poured out upon a meal which would be otherwise readily digested. The most abundant secretion is obtained with meat, while the digestive power is greatest after a bread meal. Therefore in evaluating the results secured after a test-meal, the type of meal must be given due consideration and the results which are regarded as standard for a stipulated meal would not be used as a criterion for an entirely different sort of meal.

The amount of material recovered when the stomach tube is passed will depend to some extent upon the reaction of the contents, since we know that when digestion has reached a certain point and when the food has been churned into a homogeneous mass (chyme) of the proper reaction, the propulsion of portions of this mass against the pyloric sphincter will result in the opening of the sphincter. Contact of this acid material with the duodenal mucous membrane, however, causes the sphincter to close. Different kinds of food materials are discharged differently. The discharge of fats begins slowly and continues at a slow rate over a long period of time. The discharge of carbohydrates, on the other hand, begins soon after ingestion and rapidly becomes abundant, reaching a maximum in about two hours and usually leaving the stomach empty in three hours. The discharge of proteins is about intermediate between that of fats and that of carbohydrates, beginning in about half an hour and not attaining a maximum for four hours. The rates are dependent upon the combining power of the food material for acids, the carbohydrates having practically no combining power and leaving the acid which is secreted free to open the sphincter, whereas fats inhibit gastric secretion.

It has been shown that the effect of hyperacidity is to delay the emptying of the stomach. Even after the stomach has emptied itself of food, there is more or less continuous flow of gastric juice, as has been shown by Carlson, the amount varying from a few cc. to 60 cc. per hour.

The functions of the hydrochloric acid have to do with the control of the stomach movements, such as opening the sphincter, and with the secretion of the gastric juice and bile. Its chemical functions are concerned with the digestion of proteins by assisting pepsin; with the inversion of the dissaccharides; and with the antiseptic action, which prevents the growth of bacteria, notably yeast sarcinæ, lactic acid and butyric acid bacilli, streptococci, etc. The action of pepsin and hydrochloric acid upon the proteins is in the nature of hydrolysis. The first products are acid albumin, after which in succession come primary proteose, secondary proteose, and peptone. While for many years the presence of another enzyme in addition to pepsin, *i. e.*, rennin, has been assumed, this view has met with some skepticism of late and there is a strong feeling that the clotting of milk is due to pepsin.

Characteristics.—The gastric juice is a thin, highly acid liquid, with a specific gravity ranging from 1.001 to 1.010. The amount of hydrochloric acid varies from 0.1 to 0.3 per cent. The amount of pepsin is small by actual weight. Salts are present, largely chlorides and phosphates. A certain amount of gastric juice is present even in a fasting stomach. As seen in clinical work, the gastric juice is almost always mixed with partially digested food.

TYPES OF TEST-MEALS.

The normal findings vary for the different types of meals and this should be considered in evaluating the results. The meals should be given when fasting, preferably the first thing in the morning. Only such forms of food should be given as will not interfere with the action of the indicators which are to be employed.

Ewald Meal.—This is the meal which is most frequently employed in clinical work, and has retained a degree of popularity in spite of many modifications which have been introduced. This is probably because long usage has taught the clinician just what results to expect in the average normal cases. The meal consists of a piece of white bread without crust, or a roll, to be taken with a cup of tea or a glass of water. The patient should be instructed specifically to take no butter on the bread and to use no milk or cream in the tea, and should be told to chew the bread thoroughly and to eat slowly. He should be directed to take no medicine before or after the meal. The meal should be removed about one hour after ingestion. Normally the amount recovered is between 20 and 60 cc.

Riegel Meal.—While this meal or one similar to it may be used to determine the general digestive power, it is not particularly well adapted for the purposes of chemical investigation. The meal as ordinarily given consists of a cup of bouillon, 200 gm. of finely chopped cooked beef steak ("Salisbury steak"), two slices of white bread, 150 gm. of finely mashed potatoes, and a glass of water. It is usually removed at the end of three or four hours. If the stomach be practically empty at the end of three or four hours with a small amount of contents recoverable, the motility is regarded as normal, whereas if the stomach contain a considerable amount of food at the end of four hours, the digestive power is low. The amount of acid after such a meal is usually higher than would be obtained after an Ewald meal.

Boas Meal.—This consists of 500 cc. of oatmeal soup, made by boiling a tablespoonful of oatmeal with 1000 cc. of water, reducing the bulk of the water by boiling down to a volume of 500 cc. The patient is instructed to use no cream, butter, or milk. This meal is removed in from one to two hours, or even longer. It is given as a meal which contains no lactic acid.

Dock Meal.—When it is necessary to give a meal free from lactic acid, Dock's meal would be given preference by most patients to Boas's on the grounds of palatability. It consists of a shredded wheat biscuit moistened with hot water and a cup of hot tea.

Salzer Method of Determining Motility.—The patient is directed to take a meal consisting of one ounce of roast beef or broiled lamb chop, 250 cc. of milk, 50 gm. of rice, and 1 soft boiled egg. Four hours later an Ewald meal is given, and one hour after the gastric contents are withdrawn. The presence of meat fibers is evidence of deficient propulsive activity of the stomach wall.

REMOVAL OF GASTRIC CONTENTS.

The selection of the equipment which is used for the purpose of recovering the stomach contents is a matter of considerable importance. The tube should not be of too great caliber. The outside diameter should not exceed 12 mm. It should be provided with an opening at the end and with at least one at the side about an inch above the end. This is important since when one opening becomes occluded the contents may still be secured through the other. The side opening is not so likely to become occluded by contact with the gastric mucosa as the one on the end (Fig. 76). The writer prefers to use a Pollitzer bag for the purpose of securing the necessary suction (Fig. 76). It is easier to handle than more complicated outfits and there is nothing to get out of order. The tube is simply moistened with warm water before introducing it. Though the patient may assume either the recumbent or sitting posture; the former is preferable. After reassuring the patient a towel or rubber apron should be placed over his clothing while the operator should protect himself with an apron. The patient should take out any removable artificial teeth and should be directed to tip the head forward slightly. The mouth should be only partially opened. The Pollitzer bag is compressed and attached to the tube which is then introduced over the tongue, to one side of the epiglottis. The greatest gentleness should be used. The patient should take deep sighing breaths while the tube is in the esophagus. If the Pollitzer bag be compressed as directed until the esophagus has been entered, and if pressure be then released, the wall of the esophagus will grip the tube tightly until the tube enters the stomach, when air will rush into the bag, distending it. The length of the esophagus varies, so that the black ring which is used as a guide is of little assistance. The bulb and tube should be connected by a thick piece of glass tubing, so that it will be possible to note the passage of contents into the bag.

When the contents do not appear after this procedure, certain steps may be taken to expedite their recovery. It is possible that the orifices of the tube have been plugged with pieces of food material. The bag may be detached and allowed to fill with air, when it may be re-attached to the tube and air forced through very gently, withdrawing the tube a little at the same time. Then the bag is disconnected and re-attached for the purpose of making suction. If this maneuver is unsuccessful, the tube may be withdrawn for two or three inches. It is the writer's opinion that for the average person, the black mark should be placed farther down the tube, that is, the tube should not be introduced quite so far as the mark indicates. Gentle massage can be applied over the stomach and is always helpful.

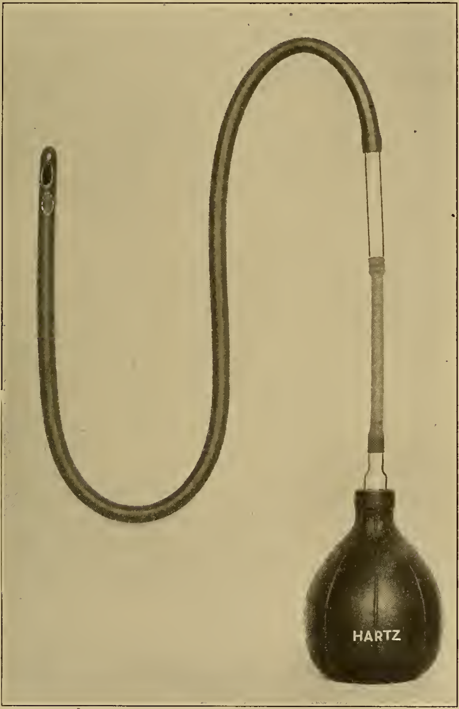

FIG. 76.—Aaron's improved stomach tube and bulb.

Contra-indications to the Use of the Stomach-tube.—There are certain rather generally accepted contra-indications to the use of the tube. It should not be passed in a case of aneurism, nor in one of uncompensated heart disease. Recent severe gastric hemorrhage, especially if from a gastric ulcer, or perforation of stomach, constitute contraindications.

Obtaining Gastric Contents with the Duodenal Tube and the Fractional Method of Examination.—An Einhorn or Rehfuss tube (Fig. 77) should be provided. There should be a glass syringe of 20 cc. capacity, which may be attached to the free end of the tube.

The patient may be given a meal of the Ewald type, two slices of bread and ten ounces of tea or water. When this is employed, the crust should be removed from the bread, and especial precautions should be taken to see that mastication is thorough. At the close of the meal the tube is introduced. Two or three ounces of tea may be reserved and may be taken with the tube to facilitate its passage. The patient should be in the sitting posture. He is directed to tip the head back and the metal olivary body is slid into the pharynx over the dorsum of the tongue. Usually its further passage may be accomplished by directing the patient to swallow repeatedly. The tip usually reaches the lumen of the stomach before the 50 cm. mark on the tube passes the incisor teeth. The contents are now obtained by aspirating with a syringe. A small quantity, *i. e.*, a little over 5 cc., may be removed every fifteen minutes until the close of digestion. The tube may be secured in place by attaching to it one end of a strip of adhesive tape, fastening the other end of the adhesive tape to the face. Each specimen should be examined separately for the amount of free HCl and total acidity. The small residues remaining may be pooled and utilized for the tests for lactic acid, blood, bile, and for microscopical examination. In making the titrations, it is desirable to use 1 cc. quantities of contents and $\frac{N}{50}$ sodium hydrate, multiplying the results by 20 to reduce to the usual terms (*i. e.*, number of cc. of $\frac{N}{10}$ NaOH required to neutralize acid in 100 cc. of gastric contents). If desired, a cup of hot beef bouillon may be given instead of the Ewald meal. If bouillon be used, it should be remembered that the acidity curve is likely to be higher. Some workers prefer to introduce the tube when the bouillon is given, using the bouillon as a lubricant to assist in the passage of the tube.

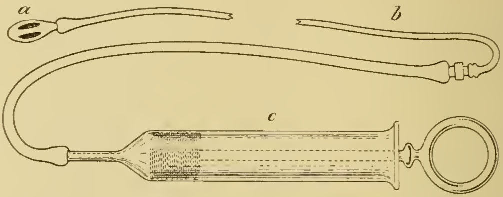

FIG. 77.—Rehfuss tube. *a*, metal olivary body; *b*, rubber tubing; *c*, glass syringe.

From the chemical determination made on the successive fractions of gastric secretion, a curve may be constructed in which the acidity per cent. (the number of cc. of $\frac{N}{10}$ NaOH required to neutralize acid in 100 cc. of contents) is placed on the vertical line, and the time intervals are spaced along the horizontal base line.

Rehfuss states that with this tube it is possible to determine the end-point in gastric digestion by failure to aspirate material where the patient is placed in different position; by the character of the last samples removed which are thicker and smaller in quantity; by the character of the sound produced by insufflation and heard by auscultation over the stomach; and by lavage. He and his co-workers studied the curve in presumably normal individuals and found that there were three types of curves, which they term respectively isosecretory, hypersecretory, and hyposecretory. In the first there is a steady rise, a high point of total acidity of about 60 sustained for about half an hour, and then a gradual decline with total disappearance of food residues in from two to two and one-half hours. The hypersecretory type shows a rapid response with increase in acidity to a high point of 70 or 100 or over, and a slow decline. In the hyposecretory type the curve is similar to the isosecretory with a slower response to stimuli, a slow ascent to the curve, and a high point of 40 to 50. They feel that the technic commonly employed for determining gastric function is entirely inadequate, inasmuch as it indicates but one phase in a constantly changing cycle, and because that phase is by no means always the high point in the digestive curve.

ROUTINE EXAMINATION.

The routine examination should include the following: (1) Note the type of meal and the time which elapsed between ingestion and removal. (2) Macroscopic characteristics, especially amount, color, odor, amount of food material; amount of mucus, if any; separation into layers; presence of recognizable blood or bile. (3) Qualitative chemical tests, free HCl; blood and bile, if macroscopic appearance or patient's history arouse suspicion on either score; lactic acid. (4) Quantitative chemical tests, free HCl; total acidity; combined acids, when free HCl is absent. (5) Microscopic examination.

Order of Procedure.—After noting the data about the type of meal and the time it remained in the stomach, the macroscopic appearance should be studied and recorded. The contents should be allowed to stand in a conical glass for at least fifteen minutes. Then a drop is placed on each of two or three slides for microscopic study. The major portion is filtered, preferably through gauze or filter paper while a small quantity, five or ten drops, is filtered through moistened filter paper and is used for the lactic acid test. While filtration is proceeding, the microscopic examination may be made. When this is completed, the filtrate should be ready for the chemical tests.

Gross Appearance.—The gross appearance often furnishes information of great importance, and its study should not be neglected. The color, separation into layers, and amount are of particular significance.

Amount.—If no food be found after an Ewald meal which has been removed at the end of one hour, this fact may be accepted as evidence of hypermotility. Normally, there is a return of from 20 to 60 cc. Large quantities (200 to 300 cc.) point to gastrosuccorrhea, motor insufficiency, or an obstruction at the pylorus. Motor insufficiency may be seen in anemia, pulmonary tuberculosis, and acute infectious diseases. Obstruction at the pylorus may be produced by carcinoma, syphilitic disease, or pressure from without the stomach, such as that produced by malignancy, adhesions, etc.

Color.—After an Ewald meal the color of the fluid portion of the contents is white or grayish-white. Bright red blood may come from the nose or throat, or merely from an injury inflicted in the passage of the tube. When due to trauma, the amount is usually small. A gastric or esophageal ulcer may be the source of blood. When the blood has been shed in the stomach, or has been swallowed, it is usually thoroughly mixed with the contents and is brown in color due to the action of the acid upon hemoglobin. When so mixed and changed in color identification should be completed by means of tests for occult blood.

Occasionally the contents show a greenish-yellow color due to the presence of bile, which may have been forced back from the duodenum in retching. Gmelin's test for bile may be employed to confirm this finding. The presence of bile on several occasions indicates a stenosis of the duodenum below the opening of the common bile duct. A green color is not always due to bile, however.

Odor.—Usually the gastric contents have a slightly sour odor in normal cases after the meals ordinarily given. A markedly sour odor is caused by butyric or acetic acid, or the presence of drugs, such as valerianic acid. The odor of putrefying flesh is sometimes noted in cases of gastric carcinoma where there is much necrotic tissue. A fecal odor may be detected where the intestinal contents have been forced back up into the stomach in intestinal obstruction.

Amount of Food Material.—The relative proportion between food material and fluid should be noted.

Presence of Mucus.—If mucus from the upper respiratory tract be swallowed, it may be seen in the contents and may be recognized by its dark color, due to the presence of inhaled carbon if the individual be a city dweller and by its stringy appearance when the stirring rod is drawn through the contents, while stomach mucus is clear, sago-like in appearance, and forms a homogeneous layer on top of the test-meal.

Layers.—Usually the contents separate into two layers. When placed in a cylindrical graduate, it will be seen that in normal cases there is an approximately equal amount of granular food material below and comparatively clear fluid above. In cases of hypersecretion, the fluid portion is relatively greatly increased, while with hyposecretion, the consistency is thick since there is little fluid. In cases of pyloric stenosis and catarrhal gastritis, there is usually a considerable quantity of contents of which on standing separates into three layers, the upper one being mucus, the middle clear fluid, and the bottom food particles. In severe catarrhal processes, the abundant mucus may carry up food particles into the upper layer.

Qualitative Chemical Tests.—*Free Hydrochloric Acid.*—This may be tested for very simply with either *Congo-red* or with *dimethyl-amino-azobenzol.* The first indicator will give a blue color in the presence of free hydrochloric acid, the latter a red. In using either indicator, a cubic centimeter or so of the stomach contents to be examined may be placed in a test-tube or evaporating dish and to it may be added a drop or two of the indicator in solution.

Dimethyl-amino-azobenzol solution (Töpfer's reagent) may be prepared by dissolving 0.5 gm. of the dry indicator in 100 cc. of 95 per cent. alcohol. Congo red is made by dissolving 0.5 gm. in 90 cc. of water and then adding 10 cc. of 95 per cent. alcohol.

It is sometimes convenient to employ these indicators in paper form. Papers may be prepared by dipping filter-paper in a solution of the indicator. The paper is allowed to dry and is cut into small pieces, which may be preserved in bottles for a considerable period of time. In employing papers for a test, a drop of the stomach contents is touched to the paper when the characteristic color change should appear in the presence of free hydrochloric acid.

Congo red gives a violet color with organic acids.

Günzberg's reagent is preferred by some since it gives no color change whatever with organic acids. The reaction is striking and unmistakable. The disadvantages are, that the solution does not keep indefinitely, and that the performance of the test requires a little more time. The procedure is, to dry two or three drops of the reagent in a clean porcelain evaporating dish or porcelain spoon very slowly and carefully over a low flame, taking especial care to prevent charring. With a glass rod a small portion of the stomach contents is touched to the film formed by the dried reagent. The dish is again warmed gently when a purplish-red color will appear if free hydrochloric acid be present. Günzberg's reagent is prepared by dissolving 2 gm. of phloroglucin and 1 gm. of vanillin in 100 cc. of 95 per cent. alcohol.

Boas' reagent is preferred by many because of its greater stability. The test is performed in the same way. The reagent is prepared by dissolving 5 gm. of resorcinol and 3 gm. of sucrose (cane-sugar) in 100 cc. of 50 per cent. alcohol.

Lactic Acid.—The test for lactic acid which should be given preference in the clinical laboratory is Kelling's. Two test-tubes should be chosen which are of about the same bore. One is filled with distilled water and in it are placed two or three drops of 5 per cent. aqueous solution of ferric chloride. The color should be a very faint but unmistakable yellow. After thorough mixing the solution is divided equally between the two tubes. One tube serves as a control on the color. To the other are added two or three drops of the stomach contents, previously filtered through filter paper. When lactic acid is present, a yellow color appears. This is more readily apparent if the tubes be held over a sheet of white paper and viewed from the top in order to look through the depth of the column of fluid.

It is preferable to free the stomach contents from substances other than lactic acid which may give the test and from substances which may interfere with the tests. A portion of the contents should be shaken up with ether. The mixture is allowed to stand for a few moments to permit its separation into layers. The ethereal portion is pipetted off. This is evaporated to dryness, the residue is dissolved in water, and the solution used in making the test.

A more convenient method for preparing an ethereal extract is with the aid of a Strauss separatory funnel (Fig. 78). It is provided with two marks, one for 5 cc. and one for 25 cc. The stomach contents are poured in to the 5 cc. mark and ether is added to the 25 cc. mark. The upper end is stoppered and the mixture is shaken vigorously for a few moments. After this it is allowed to stand long enough for the ether to separate, when the stop-cock is opened and the contents are allowed to run out until the upper level of the fluid reaches the 5 cc. mark, that is, until there are only 5 cc. of ethereal extract left in the funnel. Water is added to the 25 cc. mark. This aqueous dilution may be employed for performing Kelling's test as described above.

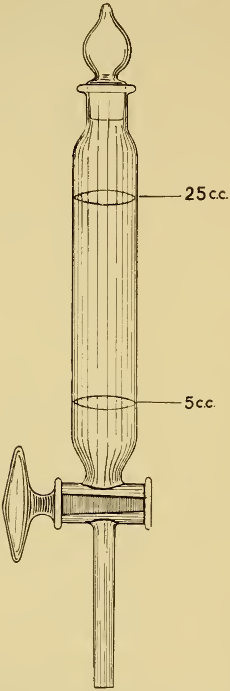

Fig. 78.—Strauss' funnel for making lactic acid test. (Aaron.)

A rough quantitative test, sufficiently accurate for all clinical purposes, may be carried out by adding two drops of 10 per cent. ferric chloride to the diluted ethereal extract in the Strauss funnel, shaking gently. It is stated (Wood) that with 0.1 per cent. of lactic acid, there will be a very intense yellow green color, while 0.05 per cent. will show only a *slight green color*.

Uffelmann's test is not recommended for the detection of lactic acid in stomach contents, because of the interference caused by organic substances.

Significance.—There are always small amounts of lactic acid present after meals which contain bread, milk, or meat. When the presence or absence of lactic acid in the contents will have weight in the diagnosis, a lactic-free meal should be given. The presence of lactic acid after such a meal usually accompanies an anacidity and is due to the fermentation which takes place in the absence of hydrochloric acid. It may be found in gastric carcinoma but also in other conditions where retention and fermentation are seen, such as benign pyloric stricture, dilatation of the stomach, and permanent anacidity.

Blood.—Blood may be recognized microscopically when it has not been digested by the action of acid. Usually, however, blood which has been in the stomach any period of time is altered and chemical tests are necessary for identification. The benzidine test may be used. In a test-tube are put 2 cc. of unfiltered gastric contents with 1 cc. of glacial acetic acid. To the mixture are added 3 cc. of a saturated alcoholic solution of benzidine. The contents of the tube are thoroughly mixed and 5 or 6 cc. of fresh hydrogen peroxide are added. In the presence of blood a deep Prussian-blue color appears.

Significance.—Blood may be seen in the stomach contents as a result of trauma inflicted in the passage of the tube. In such cases, it is usually unchanged in color, bright red, and often comes in streaks with a little mucus. It may be mixed with the stomach contents. In the latter case it may have been swallowed and have had its origin in the upper portion of the respiratory tract, in the mouth, or in the esophagus. Lesions of the stomach which may produce hemorrhage are, notably, gastric ulcer or gastric carcinoma.

Bile.—The bile tests may be carried out as directed for urine, employing a filtrate of the contents. (See page 233.)

Significance.—Bile in the stomach contents may be due to retching during the process of removing the test-meal. A green color in the stomach contents is not always due to the presence of bile, but may result from the presence of organisms containing chlorophyl.

Quantitative Chemical Tests.—The quantitative determinations usually made on the gastric contents include a determination of the amount of free hydrochloric acid, of the total acidity, and at times of the combined acids. The acids in the stomach include free hydrochloric acid, combined hydrochloric acid, organic acids, and acid salts. In the presence of free hydrochloric acid, the amount of organic acids is small. By combined hydrochloric acid is meant the hydrochloric acid which has combined with food-protein to form acid-albumin.

It is of assistance to remember the end-points of the indicators and their significance.

Dimethyl-amino-azobenzol, end-point, canary yellow; when reached means that the free mineral acids have been neutralized.

Phenolphthalein, end-point, deep red; means that the free acids, combined acids, and acid salts have been neutralized.

Alizarin, end-point deep violet; means that the free acid and salts have been neutralized.

Procedure.—Filtered contents should be used. First one of the qualitative tests should be employed to determine whether free hydrochloric acid be present or not. If absent, there should be determined only the total acidity, the combined acids, and the hydrochloric acid deficit.

When free hydrochloric acid is present, the following procedure should be followed: A burette (Fig. 54, *f*) graduated in 0.1 cc. should be charged with decinormal sodium hydrate. The sodium hydrate should be run out until the nozzle is filled and the initial reading (*A*) taken and recorded. In a porcelain evaporating dish are placed 5 cc. of the filtrate to which are added about 25 cc. of distilled water and three drops of dimethyl-amino-azobenzol solution. A bright red color should appear in the presence of free hydrochloric acid. $\frac{N}{10}$ NaOH is now added to the stomach contents drop by drop until the color becomes ochre-yellow, or until the last trace of pink just disappears, stirring the mixture in the evaporating dish with a glass rod. The free hydrochloric acid has been neutralized when this color has been reached.[1]

To the same portion of partially neutralized contents, now ochre-yellow in color, 3 drops of phenolphthalein solution (1 gm. of phenolphthalein dissolved in 100 cc. of 95 per cent. alcohol), should be added. The reading (*C*) of the burette is taken and the decinormal sodium hydrate is added while constantly stirring the mixture in the evaporating dish until the color becomes a deep pink, in fact until the maximum color change has been secured. It is not sufficient to obtain merely the first permanent trace of pink, since if this color were taken as the end-point the acid salts would not be neutralized. When the proper end-point has been secured, the reading should be taken (*D*). Determinations should be made on a second portion of 5 cc., and the average taken.

Calculation.—The results are ordinarily expressed in terms of the number of cc. of decinormal sodium hydrate which are required to neutralize 100 cc. of the gastric contents. The results obtained in the titration with 5 cc. of the gastric contents are therefore multiplied by 20.

Example.—The original reading in the burette (*A*) was 5.5. The reading when the end-point for dimethyl-amino-azobenzol was reached (*B*) was 7.5. The reading when the titration with phenolphthalein was reached (*C*) was 7.5. The reading when the end-point for phenolphthalein was reached (*C*) was 9.5.

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[1] In many texts it is directed to carry the neutralization until a canary-yellow color appears. This method has been criticized by A. L. Benedict who states that while the color change from cherry-red to canary-yellow is almost instantaneous in titrating solutions of inorganic acids, the change in the stomach contents is very gradual, due to the presence of organic acids and to the organic combination of part of the hydrochloric acid. He states that the reading for free hydrochloric acid should be taken at the point when the cherry-red tint disappears and is replaced by an orange tint. the Benedict's point is well taken and may be verified by adding to a second specimen of contents the amount of decinormal sodium hydrate ascertained by a titration made in the manner he prescribes. When a portion of this neutralized mixture is tested with Günzberg's reagent, it will be found to contain no free HCl. There may be a difference of from 10 to 15 degrees in the final end-point as determined by the methods.

B − A = 2. This multiplied by 20 gives 40, the number of cc. of decinormal NaOH required to neutralize the free HCl in 100 cc. of gastric contents.

(B − A) + (D − C) = 4. This multiplied by 20 gives 80, the number of cc. of decinormal NaOH required to neutralize total acids in 100 cc. of gastric contents.

The number of cc. of decinormal sodium hydrate required to neutralize a given amount of stomach contents when multiplied by the factor 0.00365 will give the actual amount of free hydrochloric acid in that amount of stomach contents, expressed in grams.

Combined Acids.—When desired, the amount of the combined acids may be determined by titrating 5 cc. of filtered gastric contents with $\frac{N}{10}$ NaOH, using a few drops of 1 per cent. aqueous solution alizarin as an indicator. The end-point has been reached when a deep violet has been obtained. One should become familiar with the end-point by adding a drop of alizarin to a 1 per cent. solution of sodium carbonate. Subtract this reading obtained from the reading obtained with the phenolphthalein as an indicator, multiply the remainder by 20, and the result indicates the combined acids in 100 cc. of gastric contents.

Hydrochloric Acid Deficit.—When free hydrochloric acid is absent the "HCl deficit" should be determined. Place 5 cc. filtered gastric contents in an evaporating dish and add 3 drops of dimethyl-amino-azobenzol solution. Add from burette $\frac{N}{10}$ HCl until rose-red color is obtained. Take original reading from final reading, and multiply by 20 to express the number of cc. of $\frac{N}{10}$ HCl required to neutralize 100 cc. of gastric contents.

Discussion of Method.—The method which has been given is that of Töpfer. While it is open to criticism, especially because dimethyl-amino-azobenzol reacts to organic acid in large quantity as well as to free mineral acid, and while the results may be not extremely accurate, it serves clinical purposes excellently. It is not satisfactory in the absence of free hydrochloric acid. The use of more elaborate and time-consuming methods is scarcely justified, particularly when it is remembered that the results after all test-meals are subject to variations. As has been remarked by Benedict (*Medical News*, 1904, lxxxxiv, 597), "it really makes little difference which of several indicators is used provided one understands that the results are approximate and reliable only for comparative purposes. With low hydrochloric acid and high fermentation acidity, the readings of the burette are very inaccurate though they indicate the principles of therapeutics clearly enough." Whenever it is necessary to have an accurate test made, the chyme must be incinerated.

Normal Results.—The free hydrochloric acid in a specimen removed one hour after an Ewald meal is usually between 30 and 40 (cc. of $\frac{N}{10}$ NaOH required to neutralize free HCl in 100 cc. gastric contents), representing about one-third to one-half of the total acidity. The combined acid may be added to the free hydrochloric acid to ascertain the amount of useful acid secreted, since combined acid is acid which has partially fulfilled its function of digestion. The figures for combined acid vary greatly, averaging about 10 cc. There is, however, no rigid standard which can be set down as a normal for any of the findings, since marked variations are seen even in health. The results obtained by the fractional method show that the maximum excretion of acid is reached in some cases before the end of the hour, after an hour in others. To set down the results obtained from a single determination as representing the patient's response to a meal, is open to obvious error. The figures vary with age, social condition, race and with nervous factors which may influence psychic or "appetite" secretion.

The term *euchlorhydria* is used to describe the presence of hydrochloric acid in normal proportions. The absence of hydrochloric acid is usually spoken of as *anacidity* of *achlorhydria*. *Achylia* is the condition in which neither hydrochloric acid nor enzymes are present. *Subacidity* or *hypochlorhydria* refers to a diminished secretion, and *hyperchlorhydria* or *hyperacidity* to an increased secretion of hydrochloric acid. *Achlorhydria* is seen in advanced gastritis, in the neuroses, in gastric carcinoma and in achylia gastrica, which is commonly found in pernicious anemia. *Hyperchlorhydria* may be occasionally found in presumed health. It is also found in the neuroses, in beginning gastritis, and in ulcer of the stomach. *Hypochlorhydria* is seen in subacute or chronic gastritis, occasionally in the neuroses, at times in gastric or duodenal ulcer, in early carcinoma of the stomach, in the course of acute general infections, and in advanced tuberculosis.

Microscopic Examination.—Microscopic examination is of comparatively limited usefulness but should be carried out as part of the routine examination, since through it occasional clues are obtained. A drop of the unfiltered contents should be placed on a slide and covered with a cover-slip. The field should be darkened by dropping the Abbé condenser and by partially closing the substage diaphragm, since it is impossible to study the formed elements with a well-illuminated field. The examination should be conducted at first with the low power and later with the high power dry lens. The presence or absence of the following elements should be noted: elastic tissue, muscle fibers, fat drops, fatty acid crystals, starch granules, and organisms of various types, especially large bacilli, sarcinæ, and yeast cells (Fig. 79). If testing with Lugol's solution or Sudan III be desired, a drop of the reagent may be placed on the slide next to one side of the cover-slip when a bit of filter paper may be touched to the fluid under the cover-slip at the other side, starting a current which draws the reagent under the cover-slip.

Fat drops appear as globules of varying size of moderate refractility. They stain red with Sudan III. *Fatty acid crystals* are long, needle-like crystals which melt into globules. *Sarcinæ* are readily recognized by the typical bundle-like arrangement of the cocci. *Yeast cells* may be distinguished by the ovoid outline and the budding which some of the cells show. The only bacilli of special note are the *Boas-Oppler bacilli,* which are found in large numbers when free hydrochloric acid is absent. They are long and exceedingly large rods, occurring in long, jointed chains (Fig. 80). With Lugol's solution, the Boas-Oppler bacilli stain brown, while mouth organisms of similar morphology (Bacillus maximus buccalis, Leptothrix buccalis) stain blue. The

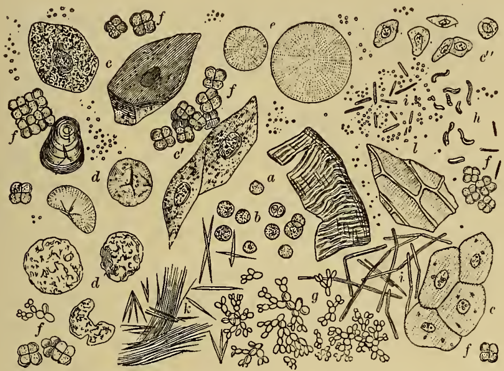

FIG. 79.—Collective view of vomited matter. (Eye-piece III, objective 8 A, Reichert.) *a*, muscle fibers; *b*, white blood corpuscles; *c*, c', squamous epithelium; c'', columnar epithelium; *d*, starch grains, mostly changed by the action of the digestive juices; *e*, fat globules; *f*, sarcinæ ventriculi; *g*, yeast fungi; *h*, forms resembling the comma bacillus found by the author once in the vomit of intestinal obstruction; *i*, various microörganisms, such as bacilli and micrococci; *k*, fat needles, between them connective tissue derived from the food; *l*, vegetable cells. (v. Jaksch.)

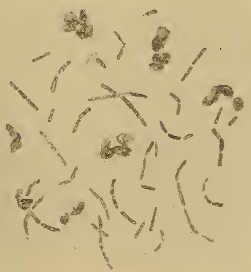

FIG 80.—Boas-Oppler bacillus. (Simon.)

Boas-Oppler bacilli are Gram-positive. The work of Galt and Iles (*Jour. of Path. and Bacter.*, 1914–1915, xix, 239) makes it appear that the Boas-Oppler bacillus is identical with B. bulgaricus, and that it is not an organism *sui generis*. It grows in the absence of hydrochloric acid, and produces lactic acid.

SPECIAL CHEMICAL TESTS.

Pepsin.—The determination of pepsin is not usually required, since ordinarily pepsin is present when there is free hydrochloric acid. When free HCl is absent, however, it is advisable to make tests for pepsin. The qualitative test, which is readily performed, serves all practical purposes.

Qualitative Test for Pepsin.—Small discs of coagulated albumin are prepared from the white of an egg which has been coagulated by boiling in a test-tube placed in a water-bath. Boiling must not be allowed to continue beyond the point of coagulation, since it may render the albumin difficult of digestion. The block of egg-white is sliced into thin layers about 1.5 mm. thick with a safety-razor blade and small discs about 10 mm. in diameter are cut out of these layers with a sharp cork borer. The discs should be preserved in glycerol. One of them should be tested with normal stomach contents to ascertain the time which is required for complete digestion and the time so determined should be noted on the bottle. To perform the test, one of the discs is freed from glycerol by thorough washing with water and placed in about 10 cc. of stomach contents. If the contents contain no free hydrochloric acid they should be acidified with enough decinormal hydrochloric acid to turn Congo-red paper blue. The tube containing the contents and disc is then placed in a warm place, preferably in the incubator at 37° C., and shaken at intervals. At the end of two or three hours the disc should have dissolved. The exact time should be compared with that determined for the particular set of discs which were employed.

Mett's Quantitative Method.—Mett's method gives approximately accurate results. Egg-white is diluted with an equal volume of water to precipitate the globulin, which is removed by filtration, using the suction pump if necessary. The filtrate is placed in a large test-tube or narrow beaker and in it are immersed a bundle of fine capillary tubes, about 2 mm. inside diameter and of convenient length. The tubes are allowed to stand completely submerged in the albumin solution until free from air-bubbles, when the vessel with its contents is placed in a bath of boiling water for from five to fifteen minutes, depending on the amount of fluid. Boiling should not be continued longer, since the coagulated egg albumen softens with prolonged heating. The tubes are removed from the vessel, the albumin on the outside is scraped off and the ends sealed with a little sealing wax. When used for tests, sections about 2 cm. long are cut off and the sealing wax removed from the ends.

The stomach contents are diluted 16 times with twentieth-normal hydrochloric acid (adding 15 cc. of $\frac{N}{20}$ HCl to 1 cc. of the gastric contents). One of the Mett tubes is put in the diluted gastric contents and kept at 37° C. for twenty-four hours when the length of the column of albumin which has been digested is measured with a millimeter scale, using a hand lens. In normal gastric juice, the upper limit of digestion is usually 4 mm. The amount of enzyme present is proportional to the square of the number of millimeters digested. For example, if one specimen digests 2 mm. and another specimen digests 3 mm., the ratio between the enzyme values based on the squares of these figures, would be as 4 is to 9.

Quantitative Determination of Pepsin (W. C. Rose's Method) (*Arch. Int. Med.*, 1910, v, 459.)—*Solutions Required.*—I. 0.25 gm. of pea-globulin dissolved in 100 cc. of 10 per cent. sodium chloride solution (using gentle heat if necessary to secure solution), and the solution is filtered.

Pea-globulin is secured by grinding about 2 handsful of ordinary garden peas (Pisum satirum), and by extracting them with 200 cc. of 10 per cent. sodium chloride solution. The peas should be freed as much as possible from their outer coating when they are extracted repeatedly with large quantities of 10 per cent. sodium chloride. The extracts are combined and filtered through bolting cloth. After standing overnight in large cylinders to permit the deposition of insoluble material, the supernatant fluid is siphoned off, saturated with ammonium sulphate and filtered. The filtrate is discarded and the precipitate, containing the albumins and globulins, is placed in a dialyzer and dialyzed in running water for three days to remove the salt and dissolve the albumins. The globulins, which remain as a precipitate, are filtered, washed in water 2 or 3 times to remove all albumin, and dissolved in 10 per cent. sodium chloride. The resulting solution is filtered until perfectly clear and neutralized to litmus by adding dilute sodium hydroxide very carefully. Dialyzation is again employed for three days to remove the salt; the precipitated globulins are filtered and dried in a water-bath at 40° C. The proteins should be preserved during the entire process of separation by the addition of an alcoholic solution of thymol and toluene. Globulins prepared in this way dissolve almost completely in 10 per cent. sodium chloride, and after acidification with hydrochloric acid, yield a turbid solution.

II. Hydrochloric acid (0.6 per cent.). This may be prepared from concentrated (36.5 per cent.) HCl by adding 2 cc. of the latter to 118 cc. of water.

Procedure.—A known amount of the gastric contents is made neutral to litmus by adding $\frac{N}{10}$ NaOH. Should a precipitate appear, it is removed by filtration. Sufficient water is then added to dilute to five times the original volume. The diluted and neutralized stomach contents are then divided into two portions, one of which is boiled.

In each of 6 test-tubes place 1 cc. of the globulin solution and 1 cc. of the hydrochloric acid solution. Allow five minutes to elapse for the development of turbidity. With a 1 cc. pipette (graduated in 0.1 cc.) place in the tubes unboiled diluted gastric contents as follows: First tube, none; second tube, 0.1 cc.; third tube, 0.3 cc.; fourth tube, 0.5 cc.; fifth tube, 0.8 cc.; sixth tube, 1.0 cc. Then add enough boiled diluted gastric juice to bring the contents to 3 cc. The table illustrates the method of setting up the tubes.

Tube No.	1	2	3	4	5	6
Globulin solution, cc.	1.0	1.0	1.0	1.0	1.0	1.0
HCl solution, cc.	1.0	1.0	1.0	1.0	1.0	1.0
Unboiled diluted gastric juice, cc.	0.0	0.1	0.3	0.5	0.8	1.0
Boiled diluted gastric juice, cc.	1.0	0.9	0.7	0.5	0.2	0.0

Incubate at 36° C. for one hour. Select the tube containing the smallest amount of gastric juice which shows no turbidity. This contains the least amount which completely digests the globulin.

Results.—The enzyme content is expressed by the number of cc. globulin solution that would be digested by 1 cc. of undiluted gastric juice. For example, tubes No. 1, No. 2, and No. 3 are cloudy, but No. 4 is clear. This means that digestion of 1.0 cc. of the globulin solution has been effected by 0.5 cc. of a 1:5 dilution of gastric contents, or by 0.1 cc. of undiluted gastric juice. If 0.1 cc. would digest 10 cc. the enzyme content is expressed as 10 units. The normal results are about 10 units. In cancer, the results range from 0 to 3. The significance of enzyme determinations will be discussed under the consideration of the findings in various morbid condition.

Quantitative Determination of Dissolved Albumin.—(Method of Wolf and Junghaus).

Solution Required:

Phosphotungstic acid (pure)	3 cc.
Hydrochloric acid (concentrated)	10 "
Alcohol (95 per cent.)	200 "
Aquæ dest., to make	2000 "

This is kept in a bottle with a rubber stopper.

Procedure.—Prepare a 1 : 5 dilution, (*A*), by adding 4 cc. of distilled water to 1 cc. of gastric juice and a 1 : 50 dilution, (*B*), by adding 9 cc. of distilled water to 1 cc. of gastric juice. Place 1 cc. of distilled water in each of 6 test-tubes. To the first tube add 1 cc. of dilution (*A*), mix well by drawing a portion of the fluid into the pipette, ejecting back into the tube, repeating 2 or 3 times, and finally place 1 cc. of the mixture in the second tube. Mix as before, carrying 1 cc. of this mixture to tube 3, mix again, and discard 1 cc.

Next prepare tube 4 by adding 1 cc. of 1 : 50 dilution (*B*), mixing the contents as before, and placing 1 cc. in tube 5. Repeat the operations until tube 6 is mixed, when 1 cc. should be discarded. The series of tubes now contain 1 cc. respectively of 1 : 10, 1 : 20, 1 : 40, 1 : 100 1 : 200, 1 : 400 dilutions. To each add 1 cc. of the phosphotungstic acid solution, layering it over the diluted stomach contents. A pearly white ring at the junction of the gastric juice with the reagent denotes a positive reaction.

The secretion must be examined immediately after its withdrawal. The test-meal should be given on an empty stomach in order to guard against the presence of residues and the patient instructed not to swallow salivary or bronchial secretions while the meal is in the stomach. The test is applicable only to gastric contents which contain no free hydrochloric acid or blood.

Results.—Normally positive reactions are obtained in dilutions from 1 : 10 to 1 : 50 in normal individuals. When present in dilutions of 1 : 100, 1 : 200, and 1 : 400, malignancy is indicated, according to the authors of the test. Friedenwald and Kieffer obtained positive results in 13.5 per cent. of 67 cases of benign achylias, and 83.9 per cent. positive or suspicious results in 106 undoubted cases of gastric cancer. Smithies found a positive result in 86 per cent. of 83 cases of gastric cancer, in 10 per cent. of 10 cases of pernicious anemia, in 17 per cent. of 17 cases of achylia gastrica.

Volatile Fatty Acids.—The fatty acids found in the stomach contents are usually butyric or acetic acid. Butyric acid may be recognized by the pentrating odor, which is like that of rancid butter. The odor of acetic acid, similar to that of vinegar, is not so readily recognized.

A test which may be employed for the volatile fatty acids is to place a few cc. of the contents to be tested in a test-tube and suspend a strip of moistened blue litmus in the mouth of the tube. The contents are heated. The volatilized acids will redden the litmus paper.

For more positive identification, 15 or 20 cc. of the contents are shaken with about 2 gm. of sodium sulphate and 50 cc. of ether. The ethereal extract is separated and evaporated to small bulk preferably before a fan at room temperature (with care to avoid explosion). The residue is divided into two portions which are treated as follows:

One portion is dissolved in a few drops of water and a bit of calcium chloride added. *Butyric acid* will separate, since it is insoluble in calcium chloride, floating to the top as small fat drops, which will have the characteristic odor of rancid butter.

The second portion is dissolved in a small quantity of water and neutralized with a 2 per cent. solution of sodium carbonate. A few drops of 5 per cent. ferric chloride solution are added, when a blood-red color appears if *acetic acid* be present. Upon boiling, basic ferric acetate separates out as a brownish-red precipitate.

Significance.—Volatile fatty acids are not produced in the stomach under normal conditions, but may occur when there is stagnation and absence of free hydrochloric acid permits fermentation.

Mercury.—Gastric contents may be examined for mercury by the method of Lee and Vogel (see page 266).

Fragments of Tumor Tissue.—Such bits of suspicious material should be fixed properly, sectioned and suitably stained. Occasionally, the findings of carcinomatous material has furnished the final evidence in making the diagnosis of gastric carcinoma, but as a rule the pieces are too small for satisfactory examination.

Absorptive Power.—The absorptive power of the stomach may be determined by giving the patient a few grains of sodium or potassium iodide in a gelatin capsule. After filling, the outside of the capsule should be carefully cleaned to remove the salt. The iodide will be excreted in the saliva promptly in normal individuals. Specimens of saliva, obtained at intervals of two or three minutes, should be treated with a few drops of starch solution and a few drops of nitric acid. A dark-blue color denotes the appearance of iodide. In normal individuals, this is seen in from five to ten minutes. The test is not of great practical value, since occasionally the absorption is rapid even with chronic gastritis or dilatation of the stomach.

SAHLI'S DESMOID TEST.

Sahli has devised a method for testing the digestive power of the stomach, which is referred to as the desmoid reaction. In the center of a piece of rubber dam is placed 0.05 gm. of methylene blue. The dam is wrapped to form a small sack or bag, taking pains to keep the methylene blue inside the pouch, and is tied securely with No. 00 raw catgut which has been rendered pliable by prolonged soaking in cold water. The excess of rubber above the catgut is cut off. The capsule is washed carefully and tested in water to determine that there is no leakage of methylene blue.

The capsule is swallowed at the close of a full meal. Beginning two hours later the urine is examined for green color at thirty-minute intervals. When the characteristic color appears in six to twenty hours, the gastric juice contains sufficient hydrochloric acid and pepsin to dissolve the catgut and liberate the methylene blue. Sahli claims that the pancreatic juice is incapable of digesting the catgut.

FINDINGS IN VARIOUS STOMACH DISEASES.

Gastric Ulcer.—The diagnosis rests upon a careful physical examination and painstaking study of the symptoms with the confirmatory evidence furnished by the *x*-ray and the laboratory. Usually there is an active secretion of gastric juice of high acid content. This finding, however, is not by any means pathognomonic. Also indicative of ulcer is the presence of blood, especially if it be altered, and further evidence is afforded by finding blood in the stool after the patient has been placed upon a suitable diet (see page 314). It must be noted that all of these findings may be given by a carcinomatous ulcer in its early stage.

Dilatation of the Stomach (*Gastrectasis*).—The findings on physical examination of the lower border of the stomach far below the usual level and of a succussion sound long after the stomach should have emptied itself, are of prime importance. Epigastric bulging may be seen. Frequently there is a history of vomiting large quantities of sour-smelling food. Usually a large quantity of contents is obtained after a test-meal, and unless stomach lavage be performed before the test-meal, residue of food taken during the preceding twenty-four or even forty-eight hours may be found. The acidity is variable, a low acidity being found in some cases, an hyperacidity in others. When the amount of free hydrochloric acid is low, lactic acid and the other organic acids may be found with many bacteria, especially sarcinæ and yeasts. The amount of urine is usually low and the chlorides are often diminished.

Carcinoma of the Stomach.—The history and physical findings are of primary importance and the results of the test-meal examinations must be regarded only as part of the evidence. Delayed motility is to be expected when the tumor is situated at the pylorus. Free hydrochloric acid is absent and the total acidity is diminished in many cases. These findings are by no means constant, however, since continued observation has shown a normal amount of acid in the early stages in many cases. The presence of lactic acid depends, of course, upon the lack of free hydrochloric acid, as does the presence of the Boas-Oppler bacilli. Neither of the latter findings however, can be regarded as belonging exclusively to the picture of gastric carcinoma. Pepsin is diminished or absent. The amount of albumin in the contents may be increased. With ulceration, blood may be found in the contents and demonstrated in the stools. Occasionally fragments of tumor tissue are found in the water returned after lavage. Unfortunately in the early stage of a carcinoma, particularly of one which has developed upon an ulcer as a base, the test-meal shows nothing which may be regarded as characteristic.

Achylia Gastrica.—While rare, this condition is of importance, especially from a diagnostic standpoint. While seen most often in pernicious anemia, there is considerable possibility of confusing it with carcinoma of the stomach in differential diagnosis. The food returned in the test-meal shows no evidence of digestion. Free hydrochloric acid is absent, the total acidity is low, and the ferments are diminished. Lactic acid may be present, though only in small amounts. Often motility is normal. Repeated examinations of the contents should be made before the diagnosis is regarded as established.

Continuous Hypersecretion (*Gastrosuccorrhea, Reichmann's Disease*).—In addition to the objective symptoms, repeated analyses of gastric content are of value. After lavage at night, there is found in the fasting stomach on the following morning a considerable quantity of gastric juice of high acid content containing no food remnants, yeasts, or sarcinæ. The hydrochloric acid content is greater than normal with relation to the total acidity. After test-meals, the free hydrochloric acid content is increased and starch digestion diminished. Before making a diagnosis, such findings should be obtained on a number of occasions, excluding conditions which may cause a mere hyperchlorhydria and demonstrating the secretion of a considerable quantity of highly acid digestive juice without food stimulus.

Chronic Gastric Catarrh.—The amount of contents removed after an Ewald meal is normal or increased. After the condition has existed for a long time, there is a diminished total acidity and the free hydrochloric acid is low in amount or absent. The food material shows little evidence of digestion. Considerable mucus is present in many cases, often enough to render filtration impossible. Pepsin is present, though usually diminished in amount. Starch digestion ordinarily proceeds well so that there will be little erythrodextrin and much achrodextrin. The motor function usually is not greatly impaired.

The Gastric Neuroses.—Since this term is used to describe a number of vague symptom complexes, it is difficult to describe the gastric findings with exactitude. Before making a diagnosis of gastric neurosis, organic diseases must be excluded beyond question of doubt. In a given case of functional neurosis, the findings are often variable, since at times an anacidity, and at times an hyperacidity, is found. Indeed gastro-enterologists hold that only one type of case should be included under this classification, that is, one where gastric analyses show a more or less constant variation in gastric secretion, motility and sensation. While the acid findings are variable, the ferments are usually constant. Mucus is seldom seen in any quantity.

EXAMINATION OF DUODENAL CONTENTS.

Since the introduction of the duodenal tube by Einhorn it has been possible to secure the duodenal contents for examination. The practical value of examinations is not entirely settled, since there has not been a sufficient accumulation of data to permit competent judgment.

Obtaining the Duodenal Contents.—A Rehfuss or an Einhorn tube may be employed. Robinson has encountered difficulty with the Rehfuss tube, which is of relatively large size with slits on the side, because it occasionally was plugged by the walls of the pylorus during its passage so that secretion could not be obtained, and recommends making a small hole in the end of the metal olivary body. He gives the following explicit description of the method which he used for introducing the tube: "The tube was passed as a rule on a fifteen-hour fasting stomach. The tip was introduced into the mouth when the patient was lying down. The patient then swallowed, and as soon as the tip passed into the esophagus he assumed the sitting posture, being requested not to swallow any more and to expectorate all saliva. By supporting the tube and having the patient breathe slowly and deeply, the tip slowly passes down the esophagus into the stomach. The tubes are marked at 35 cm., 50 cm., 60 cm., 70 cm., etc., from the tip. When the 35 cm. mark passes the teeth, the tip is at the cardiac orifice. The patients were then placed on their right side. The tip then, together with what secretions are present, gravitates toward the pylorus. No secretions reach the pylorus. The distance from the pylorus to the mouth is indicated by the second mark (50 cm.) on the tube. Specimens were then obtained every ten minutes until the tip passed through the pylorus into the duodenum. This took, in normal cases, about thirty minutes."

To determine whether or not the tube really is within the lumen of the duodenum, Einhorn gives the following points: If upon aspiration, the fluid is obtained quickly, looks watery, and shows free hydrochloric acid, then one may know that the tube is in the stomach. If on the contrary, only a little material of neutral or alkaline reaction and of yellow color be obtained, it is duodenal fluid. When gastric anacidity is present, some confusion may arise, but when the fluid comes very slowly, the chances are that it is duodenal fluid. To settle the question, the patient may be given some fluid to drink which he has not had recently, such as milk colored with raspberry syrup. If the fluid obtained by immediate aspiration be clear and contains no milk, it is duodenal fluid, while if colored milk be obtained, the tip of the tube is in the stomach and not in the duodenum. The *x*-ray may be used to determine the exact location of the tube.

Normal Appearance of Duodenal Contents.—Normally, the duodenal contents are clear, amber-yellow in color, and neutral or alkaline in reaction. Ordinarily about 15 cc. may be aspirated at first and then about 10 cc. every ten or fifteen minutes. Free hydrochloric acid is not present normally. Upon standing for several days the amber color will change to green, due to the oxidation of bilirubin to biliverdin. Microscopically only a few leukocytes, a few epithelial cells, and a few bacteria are seen in normal cases.

Specimens should be examined *macroscopically* for gross characteristics, including color and the presence of mucus. *Chemically* they should be examined for free hydrochloric acid, total acidity, lactic acid, blood, bile, and possibly for ferments. *Microscopically,* search should be made for red blood cells, leukocytes, epithelium, bacteria, and gall-stone detritus. The technic for these examinations has been given in the section on stomach contents (q. v.) except for the tests for ferments which follow.

Tests for Ferments.—The tests for *trypsin* are not especially satisfactory. Myers and Fine give the following as fairly satisfactory tests for amylase and lipase:

Amylase.—Six test-tubes are placed in a rack. Into all but the first is placed 1 cc. of distilled water. Into both the first and second tubes is put 1 cc. of duodenal juice with a 1 cc. Mohr pipette (graduated in 0.1 cc.). The duodenal contents and water in the second tube are mixed thoroughly by drawing them into the pipette and ejecting a number of times, when 1 cc. of the mixture is placed in the third tube. The contents of this should be mixed thoroughly as before and 1 cc. put in the fourth tube. In the fifth tube, the process should be repeated, and 1 cc. of the mixture discarded. The sixth tube contains no duodenal juice and serves as a control. The tubes now contain respectively 1 cc., 0.5 cc., 0.25 cc., 0.12 cc., and 0.06 cc. of duodenal contents. All six tubes receive 5 cc. of a 1 per cent. solution of soluble starch and are placed in the incubator at 38° C. for thirty minutes, when they are immediately filled with cold water. A few drops of $\frac{N}{10}$ iodine (or Lugol's solution) are added and the tubes shaken. The tube is selected which shows a complete disappearance of all blue color, indicating complete conversion of the starch to achroodextrin.

Enzyme activity is expressed in terms of the number of cc. of starch solution which 1 cc. of duodenal contents is capable of digesting. If, for example, digestion is complete in the third tube (the one containing 0.25 cc. of contents) but not in the fourth tube, the activity is expressed as 20, since it is apparent that if 0.25 cc. digests 5 cc., 1 cc. could digest four times as much, or 20 cc. The activity varies between 5 and 200, the average being 40.

Lipase.—Two test-tubes are taken. Into each are placed 1 cc. of the duodenal contents. One of the tubes is boiled. Heating serves to destroy ferment activity. Both tubes receive 1 cc. of neutral ethylbutyrate, 10 cc. of distilled water and 1 cc. of toluene, and are shaken and placed in the incubator at 38° C. They should be shaken several times and at the end of twenty-four hours, the contents are transferred to two evaporating dishes. The acidity is determined by titration with $\frac{N}{20}$ sodium hydrate, using phenolphthalein as an indicator and continuing the addition of the alkali until the first trace of permanent pink. The titration result obtained with the contents of the boiled tube (control) is subtracted from that obtained with the contents of the other tube and the resulting figure taken as the measure of the lipolytic activity. Myers and Fine state that they have obtained figures varying from 0.3 to 4.3 cc., and the average has been 1.5 to 2.0 cc.

Findings in Morbid Conditions.—A cloudy alkaline duodenal secretion is significant of an inflammatory condition in the gall-bladder, bile ducts, duodenum or stomach. The cloud is due to bacteria and pus. It is to be remembered that if hydrochloric acid be present in the duodenal contents, there will be turbidity due to the precipitation of the bile salts, and that this turbidity disappears upon neutralization. The duodenal secretion shows in some cases of cholecystitis where there are numerous small stones an orange-yellow, coarse, putty-like detritus. McNeil states that in one case of chronic pancreatitis, there was a constant diminution of pancreatic ferments and an absence or only a trace of lipase; three cases of achylia pancreatica showed constant absence of the pancreatic ferments; and in so-called duodenitis, the contents showed considerable stringy mucus, numerous Gram-negative, motile bacilli, and cocci. He thought that the tube did not give any great amount of assistance in cases of duodenal ulcer.

Bacteriological Study of the Duodenal Contents.—The duodenal tube has been suggested as a means of obtaining duodenal contents for bacteriological examination, particularly for the detection of typhoid carriers. MacNeal and Chace and Hess suggested a technic for securing specimens for bacteriological examination. They recommended the use of a perforated tip which has been sterilized in water, and over which had been slipped by means of sterile forceps a gelatin capsule, soaked in 95 per cent. alcohol for several days. The tube fitted with the capsule was dipped in shellac several times until well coated. Hess recommended blowing off the capsule when it is in place in the duodenum, introducing air for this purpose with a syringe. Lyon has the patient employ an antiseptic mouth wash, but does not try to cover the capsule during its passage through the stomach.

Securing Bile by the Duodenal Tube.—Meltzer observed that the local application of a 25 per cent. solution of magnesium sulphate to the mucosa of the duodenum causes a complete local relaxation of the intestinal wall. Since the salt does not produce this effect when given by the mouth, that is, when it passes through the stomach before reaching the duodenum, he suggested its introduction into the duodenum by means of the duodenal tube, with the idea of causing a relaxation of the sphincter and the ejection of bile in cases of biliary colic and jaundice. He stated that 25 cc. of the solution would do no harm in adults while the dose in babies should not exceed 4 cc.

Lyon has adapted this method to the study of diseases of the gall-bladder and biliary ducts. The procedure which he outlined is as follows: The patient is examined on a fasting stomach. The mouth is rinsed with an antiseptic solution, for example, potassium permanganate, 1 grain to 2 ounces of water. A sterile duodenal tube, fitted with a metal tip, is introduced into the stomach and some of the fasting residuum is aspirated and placed into a sterile vessel (Specimen 1). It is examined for consistency, the presence of mucus, and free and total acidity.

When the tube has passed the pylorus, a syringe full of air is introduced into the duodenum. After the contents have been obtained and placed in a sterile bottle, 50 to 100 cc. of a 25 per cent. solution of magnesium sulphate are forced by the syringe into the duodenum or are allowed to flow by gravity and in a few minutes, gentle aspiration is started. The yellow color due to bile is usually seen in from two to ten minutes, becoming more pronounced as aspiration proceeds. When it deepens to a decided yellow, the material is placed in a sterile bottle and set aside (Specimen 2). Lyon believes that the first bile aspirated is that present in the bile-ducts, while the darker, more viscid, and more concentrated bile is from the gall-bladder. He now starts collecting another specimen (Specimen 3), which is also placed in a sterile bottle, followed by thinner and more transparent bile of lighter yellow color, which is aspirated much more slowly and intermittently. He assumes that this comes directly from the liver.

Lyon asserts that in this manner it is possible to obtain bile separately from different portions of the biliary tract, and to study each specimen cytologically, chemically, and bacteriologically. He has found the bile from the gall-bladder to vary in amount from 30 to 100 cc. The largest amount in his experience was 166 cc.

BILE IN PATHOLOGICAL CONDITIONS.

Lyon has found that the bile in various pathological conditions showed the following variations from normal.

Choledochitis.—The first bile collected was definitely different from normal in that it was more viscid and turbid, and contained an excess of flaky mucus. Cytologically, it showed pus-cells enmeshed in mucus, epithelial cells, and occasionally, red blood cells. Pathogenic organisms may be obtained on culture.

Cholecystitis.—In cholecystitis without choledochitis, the first bile collected is relatively normal, macroscopically and cytologically, but the second specimen is markedly pathological, being more viscid than bile from the normal bladder. It is turbid with flaky or stringy mucus, and cytologically shows pus cells, occasionally red blood cells and desquamated epithelium. Cultures show pathogenic organisms. Lyon recovered streptococci of various strains, staphylococci, B. typhosis, B. pyocyaneus, and Micrococcus catarrhalis.

Cholelithiasis.—With gall-stones, there is evidence of cholecystitis, but in a few cases there is in addition a gritty or sand-like sediment. In one case three small faceted stones were secured.

Obstruction of the ducts would alter the picture in each instance.

CHAPTER IV.

THE EXAMINATION OF THE FECES.

Introductory.—The examination of feces for clinical purposes requires only the simplest equipment and a small expenditure of time. Macroscopic examination alone often reveals information of great importance. All the procedures given as steps in the Routine Procedure may be reduced to very simple terms with practice and should be employed as readily as the clinician resorts to urinalysis or gastric analysis to aid him in the solution of his problems. Indeed Stitt asserts that in the tropics, the examination of the feces is of greater importance than the examination of the urine. The unpleasant features attendant upon the examination of the feces may be reduced to a minimum by observing the precautions which will be spoken of subsequently.

Apparatus Required.—Except for the cultural methods, the apparatus required is included in the list to be found in the Appendix for "Minimum Equipment for the Clinical Laboratory."

Composition of the Feces.—The feces are composed of undigested food, unabsorbed gastric, hepatic, pancreatic, and intestinal secretions, desquamated epithelium from the gastro-intestinal tract, débris of all sorts both from food material and from cells, and a large bulk of bacteria. It has been stated that the fecal excretion of an adult male will range from 110 to 170 gm. per day, and that the solid contents varies from 25 to 45 gm. With a coarse vegetable diet, the amounts both of total excretion and solid contents will be much greater. Bacteria make up a large proportion of fecal material, possibly one-third of the total by weight.

THE ROUTINE EXAMINATION.

The routine examination should include the following:

Macroscopic Examination.—Form and consistency; odor; mucus; concretions; parasites; undigested food; pus; blood.

Chemical Examination.—Reaction; presence of blood (when indicated, provided patient has been on an appropriate diet); presence of bile pigments (when indicated by history, and always when stool is of pasty color).

Microscopic Examination.—Food remnants; neutral fats and salts of fatty acid; red blood cells; leukocytes; parasitic ova; mucous particles; general character of bacteria.

METHOD OF TRANSPORTING SPECIMEN.

When a patient is directed to bring a specimen of feces to the laboratory, it is advisable to furnish him with a glass jar, about 4 inches high and about 4 inches in diameter, fitted with a screw top. A Mason jar of one pint capacity makes an excellent container, or a jelly glass with tight fitting cover may be used. He should be told that the jar is to be only partially filled. In hospital practice, specimens may be sent to the laboratory in small white enamel or granite pails provided with covers. When a bottle or container is opened in the laboratory, care should be taken in removing the top, especially when the container is almost filled with feces, since the specimen often undergoes fermentation and there may be a sudden and forcible expulsion of ill-smelling material. Care should be taken to have the specimen kept cold except when it is to be examined for amebæ, and delivered to the laboratory as promptly as possible, and to examine it at the earliest possible moment, before fermentation takes place. This is of especial importance when one desires to determine the extent of intestinal digestion, because under suitable conditions changes may take place in meat-fibers and other food remnants.

MACROSCOPIC EXAMINATION.

Macroscopic examination is of great importance. It should include a survey of the form and consistency, odor, color, the presence or absence of mucus, blood, pus, and undigested food. A search for parasites and stones should be made if clinical indications point to its necessity.

Form and Consistency.—Any deviations from the well-recognized normal should be noted. Both form and consistency are to a great extent dependent upon the diet, so that even under normal conditions the consistency may vary from that of a thin paste to a solid stool. A watery discharge is, of course, abnormal. Extremely soft stools are seen after the use of purgatives and cathartics; serous stools are seen in Asiatic cholera; hard, knotty stools (scybala), are found in constipation; and flattened, ribbon-like stools, while occasionally seen in normal people, are also seen when there is a tumor, stricture or other partial obstruction low in the intestinal tract.

Color.—The color of normal adult stool is dark brown. This is due to the presence of hydrobilirubin, which is formed from the bilirubin of the bile by the reducing action of certain bacteria. It is thought at the present time that hydrobilirubin and urobilin are identical. The character of the diet, however, has an important influence on the color. In infants, it is yellow, partly due to the milk diet and partly to the presence of bilirubin. With adults, an exclusive meat diet causes the feces to be dark, whereas after a milk diet they are light and yellow in color. Chocolate and cocoa give a dark color. Certain vegetables, notably spinach, when taken in large quantities, will give the stools a dark green appearance. Blackberries and huckleberries render stools dark brown or sometimes a greenish brown, due to the action of alkaline contents of the intestine upon the red coloring matter. Drugs may influence the color, bismuth turning the stools black, while after the use of iron, a grayish-black appears upon standing. After taking large doses of calomel, a green color may be seen, due to the presence of biliverdin which has been hurried through the intestinal tract before conversion to hydrobilirubin has taken place. With a diet which is liberal in fats, the stool may be pale yellow or even white while a clay-colored stool is seen in obstruction of the common duct when no bile gains access to the intestinal canal and when the fat has been insufficiently digested. A light color is also seen in tuberculous and myeloid disease of the intestine and in pancreatic disease. Black, tarry stools frequently contain blood from the stomach or upper intestinal tract. In such cases, there is ample opportunity for the conversion of the blood to acid hematin or other hemoglobin pigments, but when the hemorrhage is lower in the intestinal tract, it is usually impossible for this conversion to take place. Streaks of blood seen on the surface of a formed stool usually have their origin in the rectum or anus.

Odor.—The characteristic normal odor is said to be due to indol and skatol. The disagreeable odor is probably due in part to hydrogen sulphide. In fermentative dyspepsia, the stools may have a sour odor. The odor is offensive when the food is imperfectly digested.

Mucus.—Normally, very small amounts of mucus are seen. Larger amounts are found in various pathological conditions, and are indicative of intestinal catarrh. When thoroughly mixed with the feces, the source is probably the small intestine, while larger quantities, which are not so well mixed, come from the large intestine. Occasionally stools which are almost purely mucus are seen in dysentery, intussusception, and ileocolitis. In such cases there is usually more or less blood. At times, especially in mucous colitis, the mucus may form casts of the intestines, or may appear in ribbons or shreds.

Blood and Pus.—The significance of changed and unchanged blood has been referred to under the paragraph which discusses the color. Pus may be seen in ulcerative conditions of the bowel, due to syphilis, tuberculosis, dysentery, etc., or in larger quantities after a rupture of an abscess (*e. g.*, appendiceal abscess) into the intestinal canal.

Concretions.—The search for concretions is of especial importance after an attack of suspected biliary colic. The search for stones should be kept up for a period of several days after a suspected attack of biliary colic. The stool should be strained through a sieve. Several types of more or less complicated sieves are on the market, but they offer no especial advantage over a simple flour sifter or even the still simpler sieve used in the household for washing vegetables. The stool should be placed in the sieve and should be forced through with the aid of running water. The stones may be of the facetted, spherical or the mulberry types. "False gall-stones" are encountered at times after the administration of olive oil. They are merely masses of soap. Their formation has been used as a means of demonstrating the efficiency (?) of various fraudulent patent medicines (containing olive oil) sold to cause the passage of the stones. Confusion may be caused by seeds, olive pits, cherry stones, and small particles of impacted feces. For identification the stone should be ground up and chemical tests applied for the recognition of bile pigments and salts and cholesterol, the substances which compose the majority of gall-stones. (See page 233.)

Other concretions may be found. Coproliths are large, hard masses produced by the inspissation of feces in the intestines or appendix. True enteroliths are smaller and consist of an organic nucleus composed of blood clots, vegetable material, or similar substances, around which have been deposited layers of phosphates and intestinal detritus.

Parasites.—An entire parasite may be found in the stools. The hookworm and the pinworm are notable examples. Segments of the tapeworm may be found. The search for the parasites may be aided by the use of the stool sieve as described for the search of concretions. When an examination of the feces for parasites is contemplated, a vermifuge should be given followed by a vigorous purge. Embarrassing mistakes are sometimes made in the attempted diagnosis of parasites because of the confusion created by vegetable remnants which may resemble some of the smaller parasites, bits of orange and grape-fruit pulp having been taken for hookworm. Indeed, fly larvæ have been mistaken for hookworm. When search is made for oxyuris, fecal material may be obtained from around the anus. Sometimes the worm can be seen just outside the anal orifice. The expulsion of tænia necessitates fairly drastic treatment. Full doses of a vermifuge should not be given merely for diagnostic purposes. Hensel, Weil and Jelliffe recommend administering only one-third of the therapeutic doses of the extract of male fern.

Curd-like Masses.—Curd-like masses are sometimes of importance on account of their resemblance to other bodies (pus, concretions, etc.). Casein will become tough and fibrous upon immersion in 10 per cent. formalin. Lumps of fat may present a curd-like appearance and can be identified by their solubility in ether and by the fact that they stain red with Sudan III.

CHEMICAL EXAMINATION.

Reaction.—The stool is mixed in a mortar with water to form a watery paste and the reaction is taken with litmus paper. Normally it is neutral, faintly acid, or faintly alkaline.

Occult Blood.—Precautions should be taken to exclude the presence of iron, chlorophyl, meat derivatives, and mercury, any of which may give a positive reaction with benzidine. It is advisable to place the patient on a special diet from which are excluded all articles containing these substances. At the beginning of the diet a capsule containing 0.3 gm. of powdered carmine should be given. The appearance of red coloration marks the first stool of the diet. The second or third specimen passed after this, is taken for the occult blood test.

Benzidine Test.—A little of the stool should be rubbed up in a mortar or test-tube with enough water to form a paste of moderate consistency. A particle about the size of a hazel-nut meat is sufficient. With this is mixed thoroughly 1 cc. of glacial acetic acid and 2 or 3 cc. of saturated alcoholic solution of benzidin. After thorough mixing, 5 cc. of fresh hydrogen peroxide are added and the tube is corked and inverted several times. A Prussian-blue color indicates the presence of blood.

Compressed tablets of benzidin may be used with a water emulsion of feces according to the method of Roberts (see Urine, page 237). This method commends itself when the test must be performed in the patient's home or where the resources of the laboratory are not available.

For clinical purposes the benzidin test is preferred over the guaiac test, not only on the practical grounds that the benzidin solution may be kept on hand while the solution of guaiac resin must be prepared each time, but particularly because it is more delicate (5 to 10 times).[1]

Guaiac Test.—A thick paste is made of the feces by grinding up a portion in a mortar or test-tube with a little water. One cc. of glacial acetic acid is stirred into this paste. As much ether is added as there is acidified fecal material. The contents are shaken and the ethereal layer is allowed to separate. Separation can be hastened by centrifugalizing the mixture. The supernatant ethereal extract is poured into another tube.[2]

Significance.—The significance of blood in the stools has been discussed in the preceding paragraphs dealing with the color of the stools.

Bile Pigments.—*Corrosive Sublimate Test.*—A little of the feces is ground up in a mortar. A mixture is made with equal quantities of fecal paste and a saturated solution of mercuric chloride, and allowed to stand over night. Normally, only a red color develops, which is due to the presence of hydrobilirubin. A green color indicates the presence of unchanged bilirubin, which is pathological. Where the flow of bile has been obstructed completely, no color change appears.

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[1] For a discussion comparing the various test, see Kelly, T. H.: Detection of Small Amounts of Blood, Jour. Lab. and Clin. Med., 1916, i, 897.

[2] This may be used for spectroscopic examination, detecting blood by the presence of the absorption bands of acid hematin. When properly diluted, a solution of acid hematin shows four absorption bands, one each in the red and yellow, and one between yellow and green and one between the green and blue. To it are added 10 drops of a freshly-prepared alcoholic solution of guaiac resin (1 gm. resin dissolved in 60 cc. of 95 per cent. alcohol), and then either 20 or 30 drops of ozonized turpentine or 5 cc. of fresh hydrogen peroxide. Ozonized turpentine can be prepared by allowing turpentine to stand for some time in an unstoppered bottle. A blue color appears in the presence of blood. Pus constitutes a possible source of error.

Zinc Chloride Test.—A small portion of feces is rubbed up in a mortar with a little alcohol acidified with a few drops of sulphuric acid. The acidulated extract is evaporated at about 50° C. to small bulk and is then shaken up with chloroform. The chloroform extract is separated from the residue by standing, or better by centrifugalization, and to this extract is added a few drops of concentrated solution of zinc chloride. A green fluorescence appears in the presence of hydrobilirubin.

Significance of Findings.—The color of the stools is due to the presence of hydrobilirubin, which is formed by the reduction of the bilirubin furnished by the bile. Hydrobilirubin therefore should be found in normal stools and its absence points to occlusion of the common bile duct. Bilirubin, on the other hand, does not appear in the normal adult stool and its appearance points to insufficient reduction due to too rapid peristalsis, as in diarrhea, or dysentery.

MICROSCOPIC EXAMINATION.

Suspicious portions of the stool are picked out with wooden applicators or tooth-picks and placed on slides for examination, or a portion of the stool thoroughly mixed with water to form an emulsion. A portion about the size of a hazel-nut is transferred to a mortar, and is triturated with enough water to form a thin paste. Drops are then transferred to slides and subjected to treatment with various reagents. For examining feces, large object slides (2 by 3 inches) are convenient, giving a large area for study. The directions which have been given in describing the microscopic examination of both urinary sediments and gastric contents should be observed here. In brief, the field should be well darkened.

Slides may be treated as follows:

1. One drop is covered with an object slide and is examined in turn with the low and the high-power dry lenses. The number and condition of the muscle fibers should be noted, paying especial attention to the presence or absence of striation. One should also look for parasites, ova, red blood cells, mucus particles, fatty acid crystals, salts of calcium, drops of neutral fat, and food remnants.
2. The next drop is treated with one or two drops of saturated solution of Sudan III (made with 70 per cent. alcohol) and is covered with a cover-slip. The fat droplets stain red.
3. To this drop is added a drop of Lugol's solution. After thorough mixing, it is examined for starch cells, cysts of endameba, yeast-cells, and fungi.
4. To the last drop is added 36 per cent. acetic acid. The preparation is heated gently. The food masses are broken up, the soaps are converted to fatty acids, and connective tissue is rendered transparent.

Undigested Food Remnants.—*The food remnants* which may be seen in the feces include meat fibers, connective-tissue fibers, fat droplets, fatty acid crystals, or soaps, plant cells, starch granules, and detritus.

Practically all formed elements in the feces are colored yellow or brownish-yellow unless, of course, the flow of bile has been shut off. (Fig. 81.)

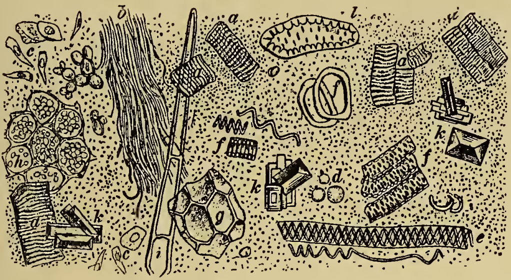


FIG. 81.—Collective view of the feces. (Eye-piece, III; objective 8 A, Reichert.) *a*, muscle fibers; *b*, connective tissue; *c*, epithelium; *d*, white blood corpuscles; *e*, spiral cells; *f*, fitted ducts; *g*, cork cells; *h*, parenchyma with starch; *i*, plant hair; *k*, triple-phosphate crystals in mass of various microörganisms; *l*, stone cell. (v. Jaksch.)

Meat fibers appear as more or less cylindrical bodies with rather square ends. When digestion is not complete, cross-striation may be made out. *Connective tissue* is recognized by the reticulum of white fibers. *Vegetable and plant cells* may occur in a variety of forms. Only experience will enable the worker to familiarize himself with the different types. Reference to the illustration will serve to present some of the more common types including stone cells, spiral vascular tubes, vegetable hairs, large potato cells, and the vegetable cells containing chlorophyl which the inexperienced are so apt to mistake for parasitic ova. *Fat* is seen in the form of refractile globules of varying size which stain red with Sudan III. Fatty acid crystals are seen (Fig. 82). They are delicate pointed needles, which melt with heat to form globules and may be identified by their solubility in cold alcohol. The soaps appear in the form of somewhat coarser needles. The greater pro-

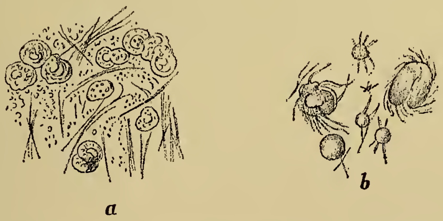


FIG. 82.—Crystals of fatty acids. *a*, mingled with mucus; *b*, around fat droplets in an infant's stool to which glycerin has been added. (From Schmidt and Strasburger.)

portion of the soaps are calcium salts, which are insoluble in alcohol though the salts of the alkaline metals are soluble in this reagent. The soaps may also appear in the form of irregular scales, colored yellow with biliary pigments (Fig. 83). Small particles of *casein* or *coagulated albumin* may be seen, also colored yellow.

The microscopic examination is of great importance from the standpoint of detecting parasitic ova, and the student should have ample practice in examining normal feces if for no other reason than to be able to recognize vegetable cells when he sees them, so that he will not confuse them with parasitic ova.

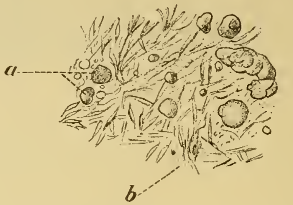


Fig. 83.—*a*, fatty acid amorphous masses; *b*, soap crystals. (From Schmidt and Strasburger.)

Red Blood Cells.—Red blood cells may be seen in the feces, when the loss of blood is great or when the hemorrhage is from a point low in the intestines. Blood shed in the stomach, small intestines, or upper portion of the large bowel is usually hemolyzed. Red blood cells may be microscopically identified by the characteristics which have been described in the chapter on Urine (see page 229).

Leukocytes.—Conditions which lead to the appearance of leukocytes in the feces have been referred to previously under the discussion of the gross appearance.

Mucus.—Mucus appears in more or less homogenous or stringy masses with faint contours. Upon addition of acetic acid, precipitation occurs, so that irregular linear markings appear. The condition in which mucus is seen in the stools have also been discussed in the paragraph on the gross appearance of stool.

Crystals.—Crystals in the feces have little significance. Triple phosphates crystals are occasionally seen especially when the specimen has been allowed to decompose partially or has been mixed with urine. Charcot-Leyden crystals are found with many of the intestinal parasites. Stitt states that while they are practically always absent from bacillary dysentery stools, they are not infrequently found in stools containing ameba. Crystals of fatty acids and soaps have been discussed. Cholesterol crystals are rare (Fig. 84). Crystals of bismuth suboxide may be seen after the administration of bismuth salts (Fig. 85). They are dark, even black, in color and irregularly rhombic in form. Occasionally hematoidin crystals are seen after hemorrhages. Calcium oxalate crystals are found at times.

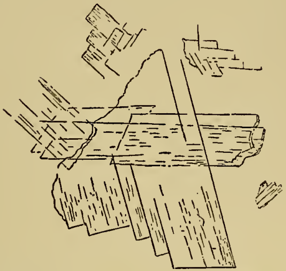


Fig. 84.—Cholesterin crystals. (Simon.)

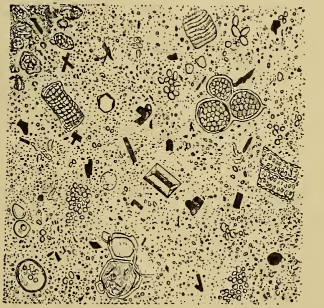


Fig. 85.—Sulphide of bismuth crystals in the feces. (v. Jaksch.)

Search for Parasitic Ova.—This may be conducted with slides prepared directly from the stool or from the watery emulsion of the stool. At times, however, ova cannot be found when there is every reason to believe that they are actually present. In such cases, it is desirable to use some method for concentrating the ova and for freeing the stools of other substances. Methods are given in the section of this chapter which deals with intestinal parasites.

GENERAL BACTERIOLOGICAL EXAMINATION.

For direct study, the bacteria are separated by centrifugalization. A small portion of feces is ground up in a mortar, as for the microscopic examination. The emulsion is placed in a centrifuge tube and centrifugalized for a short period of time. The bacteria remain in the supernatant clear fluid, while the solid portion goes to the tip of the centrifuge tube. The supernatant fluid is poured into another centrifuge tube and to it is added twice its volume of 95 per cent. alcohol. This is centrifugalized and a sediment is obtained which contains the bacteria. The sediment is taken up with a pipette and placed upon a slide. After an even spread is made, it may be dried, fixed and stained with Gram's stain. The organisms will appear as Gram-positive (*i. e.*, are purple in color), or Gram-negative. In normal adult stools the cocci will predominate over the Gram-positive bacilli. The Gram-positive bacilli are more numerous on a meat diet.

Detection of Tubercle Bacilli in the Feces.—Blood stained bits of feces or bits of mucus should be selected and smears made. The smears should be stained with carbol-fuchsin and decolorized with especial care to rule out smegma bacilli, as directed in the chapter on Urine.

Another method is to treat the feces as directed in the following paragraphs on the "Method of Isolating Tubercle Bacilli from the Feces" up to the point where the sediment is ready to be inoculated into culture tubes. This sediment should be washed after centrifugalization by pouring off and discarding the supernatant fluid. To the sediment add absolutely fresh and sterile distilled water. After mixing the sediment thoroughly to secure the solution of the salts which it may contain, the tube is centrifugalized again, the supernatant fluid is decanted, and the sediment is placed on a slide. It may be necessary to add a drop of Mayer's egg-albumen to make the sediment adhere to the slide.

The search for tubercle bacilli in the stools is far from satisfactory. Even when acid-fast bacilli are found, there is room for doubt as to whether the bacilli are tubercle bacilli or smegma bacilli, and as to whether they originated from the intestinal tract or were swallowed with sputum.

Method of Isolating Tubercle Bacilli from the Feces.—The feces are collected in wide-mouthed jars or bottles, and are diluted with three volumes of freshly distilled water. The material is mixed thoroughly with the water and filtered through several thicknesses of gauze to remove the solid particles. The filtrate is saturated with sodium chloride and allowed to stand thirty minutes, at the end of which time there will be found a floating film containing all the bacteria. This film is scooped up with a spoon and placed in a wide-mouthed bottle with an equal quantity of normal sodium hydrate. The bottle is stoppered, shaken vigorously, and then kept at a temperature of 38° C. for three hours. During this time it is shaken frequently, and at the end of the time it is neutralized to litmus with normal hydrochloric acid. This is centrifugalized and the sediment inoculated into several tubes of Petroff's or other suitable medium.

Other Bacteria in the Feces.—More extended bacteriological study of the feces requires special media and the resources of a trained bacteriologist; the methods do not lend themselves well to execution in a small clinical laboratory. Therefore, only directions for the detection of organisms of the typhoid and colon groups will be given here.

Isolation of Typhoid Bacilli.—The feces are obtained in a clean vessel and as free from outside contamination as possible. Two or three loopsful are taken up with a bacteriological loop and mixed with nutrient bouillon to make a dilute emulsion. If the stool be fluid, dilution is unnecessary. Thoroughly hardened Endo's or Krumwiede's brilliant green medium, or eosine-methylene-blue agar, is streaked and the plates incubated from twenty-four to forty-eight hours. A number of suspicious colonies (5 to 10) should be fished out with a platinum loop and inoculated into as many tubes of nutrient broth. After six hours in the incubator, hanging-drop slide preparations are made to determine motility. Transplants should be made from tubes which show motile organisms, into fermentation tubes containing sugar broths (including saccharose, mannite, dextrose and lactose), colored with neutral red. The typhoid bacillus forms acid with lactose, and causes no change with glucose, while the organisms of the colon group form acid and gas with both glucose and lactose. In the morning, if sugar readings are suggestive, agar slants should be inoculated. The growth on the slant may be used for agglutination test with a known typhoid serum in order to complete identification.

SCHMIDT AND STRASBURGER'S TEST DIET.

The patient is placed on Schmidt and Strasburger's test diet No. 2. This comprises a daily allowance of 1.5 liters of milk, 3½ eggs, strained oatmeal gruel (prepared from 80 grams of oatmeal), 100 grams of zweibach, 20 gm. of butter, 20 gm. of sugar, 125 gm. of steak (weight before cooking), and 190 gm. of potato (weight before cooking). A capsule containing 0.3 gm. powdered carmine should be given when the diet is started. The second or third stool passed after the first appearance of carmine may be used for examination.

For this test a Schmidt fermentation apparatus is used (Fig. 86). In the bottle are placed about 5 gm. of fresh feces, if of medium consistency. More or less should be taken if the specimen be very thin or very hard. When the stools are fluid, the bottle should be filled. The stool is moistened with water, with which it should be thoroughly mixed, the bottle is completely filled with water, and the rubber stopper inserted. The first of the twin tubes is then removed and filled with water. The rest of the apparatus is inverted over this tube, which is stoppered with the rubber stopper. The entire apparatus is then turned back so that the twin tubes occupy the inverted position. The apparatus is kept in the incubator at 37° C. for twenty-four hours. At the end of this time the height of column of water in the second twin tube is noted. The amount furnishes a guide to the amount of fermentation. The bottle containing fecal material is opened and the reaction of the contents is taken with litmus.

Normal Findings.—Under ordinary conditions, little or no gas is found, and the reaction to litmus usually shows little change. It is distinctly abnormal if the last tube is filled more than one-half with water. An acid reaction to litmus and light color of the fecal mixture indicate carbohydrate fermentation. A dark color and alkaline reaction indicates putrefaction.

Microscopic Examination.—The stool obtained after the test diet should be studied microscopically, as directed in preceding paragraphs. With normal digestion, there is found only a moderate number of muscle fibers, showing no distinct striation. With normal gastric digestion the number of connective-tissue fibers would be inconsiderable. Starch granules are not found in normal stools. Fat should be present only in small quantities and in the form of droplets or soaps is a sign of deficient fat digestion. With ordinary diet and normal digestion, all sorts of vegetable remnants may be seen.

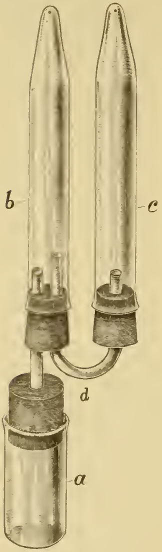


Fig. 86.—Schmidt's fermentation tubes.

FINDINGS IN THE FECES IN DISEASES.

Typhoid Fever.—There are no constant findings in typhoid fever. It is often stated that diarrhea is an early symptom. While this may be so in some localities, it is by no means invariable. Osler states that out of 1500 cases, 516 showed diarrhea and 249 constipation. As the disease progresses, diarrhea, when present, becomes aggravated and the stools take on the "pea-soup" appearance. During the course of the disease, careful watch should be maintained for the appearance of blood on account of the ever-present possibility of hemorrhage. With a severe hemorrhage, so-called "tarry stools" may be seen, which are composed almost entirely of blood.

Dysentery.—The stools are small and frequent, containing much mucus, many red blood cells and leukocytes, and a little fecal material. In the bacillary form, it may be possible to isolate one of the strains of the dysentery bacillus, while in amebic dysentery the Endamœba dysenteriæ may be found when the starch is conducted properly.

Cholera.—Small bits of mucus are seen floating in a serous stool. The frequent and profuse stools are often referred to as "rice-water stools." Microscopically, mucus, epithelial cells, and numerous bacteria may be seen. The majority of the latter are comma bacilli.

Acute Gastro-enteritis.—The stools are small and frequent, usually pasty in color, occasionally green. The fatty acids are often present in large amounts and undigested meat fibers may be found on microscopic examination. In severe cases, there may be blood and at times, an almost pure exudate of serum, leukocytes, and epithelium,

Obstruction of the Common Bile Duct.—When the flow of bile into the intestine is prevented either by a catarrhal swelling of the lower portion of the common duct or by the lodgment of a calculus in the duct, the stools present the typical "clay-colored" appearance because of the absence of biliary pigments. The odor is often unusually offensive. When the patient is allowed to eat fats, the stool frequently has a distinctly greasy appearance and shows undigested fat. On microscopic examination, the absence of bile coloration in the food remnants and the presence of large numbers of fatty acid and soap crystals are found. Chemical tests show the absence of hydrobilirubin though if the obstruction be only partial, the stool may show a certain amount of coloration. In cases of catarrhal jaundice, it is worth while from a prognostic standpoint to examine the stools for the first return of hydrobilirubin, since this is sometimes detected before the jaundice begins to lessen.

Pancreatic Disease.—The stools are often bulky and contain large quantities of undigested meat and fats. Barker states that there is more neutral fat than is seen in acholic stools. Microscopic examination shows neutral fats, many fatty acid crystals, and meat fibers showing striation plainly.

Acute Catarrhal Enteritis.—The stools vary in character depending upon the portion of the intestinal tract which is principally at fault and upon the severity of the process. The color depends upon the amount of bile, ranging from a paste-like gray color to a dark brown. The consistency is usually thin and watery though it may be like that of gruel. Small quantities of mucus are seen. If the small intestine be involved, the mucus will be bile-stained, while if the large intestine be the seat of the inflammatory process, the mucus will appear in clear masses, not stained with bile. Blood may be seen in small amounts. Portions of undigested food may be seen, especially when the small bowel is involved. Microscopically, epithelial cells, leukocytes, organisms in great number, and crystals may be found.

Chronic Catarrhal Enteritis.—When involvement of the small intestine predominates, the stools often show bile-stained mucus and small particles of undigested food. When the colon is affected, the stools are thin and contain a considerable amount of clear mucus. At times constipation is seen instead of diarrhea.

Mucous Colitis.—Large quantities of mucus are passed in the stools, sometimes comprising all of the material which is passed in a given movement. This may be seen in masses or as tubular casts. The patients often state that they have "passed a part of the bowel." Microscopically, the mucus nature is apparent. In the granular ground substance may be found cells, largely partially degenerated epithelial cells and leukocytes, with crystals of various sorts, occasionally including Charcot-Leyden crystals, and at times fine intestinal sand. In making a diagnosis it is important to remember that mucus in large quantities is also found at times in other pathological conditions, notably cancer of the bowel.

Intestinal Ulcers.—As a rule, diarrhea is present, though this is not always the case. The three cardinal evidences of intestinal ulcer are blood, pus, and tissue shreds.

Carcinoma of the Intestine.—There is nothing characteristic in the feces, though they may be frequent and sometimes show blood, pus, and mucus.

Intussusception.—In obstruction of the bowel due to intussusception, the stools contain blood and mucus. There is also fecal vomiting.

Thrombosis of the Superior Mesenteric Artery.—When acute, there is seen nausea, vomiting, and the passage of stools containing much blood.

CHEMICAL EXAMINATION OF GALL-STONES.

The suspected stone should be ground up and treated with a little hot water to remove the bile acids. The insoluble residue is treated with a warm mixture of equal parts of alcohol and ether. This serves to dissolve the cholesterol, and the alcohol-ether solution (extract *A*) is set aside to be tested for cholesterol.

The insoluble residue remaining after the extraction with the alcohol-ether mixture may contain bilirubin and calcium salts (residue *B*).

The ether-alcohol extract (extract *A*) should be evaporated carefully over a water-bath, or better, at room temperature. If cholesterol be present characteristic rhomboidal crystals will form. These may be placed upon a slide and identified microscopically. After covering the crystals with a cover-glass, concentrated sulphuric acid may be run under the cover. The crystals soften a little at the edges and become carmine-red in color. When Gram's iodine solution is added, the color changes to blue, red, green, or violet.

The residue *B* may be tested for bilirubin. First a small quantity of hydrochloric acid should be added. This sets the bilirubin free by breaking down the calcium compounds. Chloroform is added to extract the bilirubin. The chloroform extract is then tested for bile-pigments by the tests for bile already described in the chapter on Urine.

INTESTINAL PARASITES.

Method of Search.—The search for parasites is conducted according to the size and habits of the species in question. With the tapeworms, segments of the adult worm should be looked for in the stool. Oxyuris vermicularis may be found in scrapings taken from about the anus. With certain other species, the adult parasite is not found in the stool or is found only with difficulty, and reliance must be placed upon a search for ova. In still other cases, ova are not found at all, adults are found only occasionally, and reliance must be placed on finding the larvæ in other tissues (Trichinella spiralis).

The search for ova may be conducted with slides prepared directly from the fresh stools or from a watery emulsion of the stools, using a well-darkened microscopic field. This method is satisfactory in the majority of cases. At times, however, it is unsuccessful and in such cases the stools should be concentrated. Two methods are given here.

Method of Dock and Bass.—A portion of the feces is diluted to 10 times its volume with water. The suspension is filtered through 2 or 3 thicknesses of gauze in a funnel to remove the coarser food-remnants, especially vegetable remains. The filtrate is then centrifugalized for a time, long enough to bring the ova to the tip of the tube but not long enough to bring down all the food particles or bacteria. The exact time in a given centrifuge can only be ascertained by trial. Dock and Bass found that the time with the Purdy centrifuge was about four seconds. The supernatant fluid is poured off and discarded. The original volume is restored by adding water. Centrifugalization is repeated in the same way and the supernatant fluid is again poured off. The process is repeated a third time, after which the sediment is ready for examination.

Method of Yaoita.—By this method everything in the stool except certain resistant food elements and ova is dissolved and the sediment examined in the ordinary way.

Take from different portions of the stool about 5 small particles, each the size of a pea, and place in a test-tube with about 10 to 15 cc. of a mixture of equal parts of 25 per cent. antiformin and ether. Shake vigorously. There will be a marked evolution of gas. If the feces are hard, an emulsion should be made by stirring them with a glass rod, adding antiformin, and applying heat if necessary. Ether is then added. The fluid is now filtered through a layer of loose gauze to remove the larger food remnants and the filtrate centrifugalized for one minute. Four distinct layers are seen. The upper one is ether, colored yellow by the dissolved neutral fats and fatty acids. The second is a ring with suspended fine food particles, while the third is antiformin, colored yellow-brown or blackish-brown, containing detritus and dissolved food material. The lower layer, occupying only a portion of the tip of the centrifuge tube, contains undissolved food material (cellulose, epithelium, salts, elastic tissue, muscle fibers) and parasitic ova. The latter are affected little or not at all by the reagents. Fifty per cent. antiformin has a deleterious effect upon the eggs.

The dimensions of the parasites and ova given throughout the chapter have been taken from the work of Fantham, Stephens, and Theobald.

Examination for Ameba.—In making a search for amebæ, the stools should be obtained in a fresh condition. The examination should be conducted at the bedside when possible. It is advisable to have the stool passed into a warmed vessel containing a little warm normal salt solution. A bit of fecal material, preferably a particle of mucus, should be placed on the slide for study, with a hair between the slide and cover-slip, to prevent crushing the organisms or interfering with their locomotion. If the room temperature be below 70 or 75° F., a warm stage should be employed. An electrically heated warm stage, equipped with an automatic thermoregulating device and provided with a thermometer, is convenient. This should be kept at body temperature.

Entamœba Histolytica (Syns.: Ameba coli; Ameba dysenteriæ).—The parasite is found in the stools of patients suffering with amebic dysentery and in the pus from liver abscess associated with dysentery. Its average size is from 25 to 35μ. The eccentric and often feebly refractile nucleus is from 4 to 6μ in diameter. It is surrounded by coarsely granular body-substance which is divided into endoplasm and highly refractile ectoplasm. In the body of the parasite are found numerous foreign bodies, epithelial cells, bacteria, leukocytes, red blood cells, etc. Pseudopodia, composed chiefly of the ectoplasm, are projected by the organism. Smaller encysted forms are seen occasionally (so-called tetragena forms). Their ectoplasm is seen only when pseudopodia are projected. Red blood cells may be seen in the endoplasm. Nuclear division occurs, some of the round cysts containing four nuclei (tetra-nucleate cysts). Demonstration of the cysts is aided by emulsifying the stool with tincture of iodine, which colors the cyst red. Diagnosis is not possible without demonstration of ameboid motion. This can be observed only in fresh warm specimens. Stitt recommends staining the living ameba by tinging the fecal emulsion with a 1 per cent. aqueous solution of neutral red.

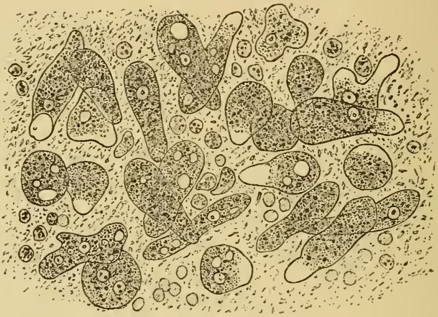


Fig. 87.—Ameba coli. (Mosler.)

The geographic distribution is wide. It is a frequent cause of dysentery in the tropics and in the southern portion of the United States. Even in the northern states and in Europe occasional cases are seen.

Entamœba Coli (Syns.: Ameba coli; Entameba hominis).—This harmless parasite is found not infrequently in the stools of normal individuals. It lives as a commensal in the upper part of the digestive tract, where the feces are still of thin consistency. With increase in the consistency of the fecal material and the change in reaction which takes place in the lower bowel, the organism becomes encysted, but when the stools of infected individuals are loose, ameboid forms may be found.

The ameboid form measures from 26 to 30μ in diameter. Ameboid movement is not marked. No distinction can be made between the endoplasm and the ectoplasm. The central'y placed and sharply defined nucleus is rich in chromatin. Ingested red blood cells are rarely seen. The encysted forms are larger, showing eight nuclei instead of the four seen in the encysted stage of the pathogenic E. histolytica.

For the purpose of differentiation between the two forms, the following table has been given by Walker:

MOTILE STAGE.

Entamœba histolytica.	*Entamœba coli.*
Appearance hyaline.	Appearance porcellaneous.
Refractiveness more feeble.	Refractiveness more pronounced.
Movements active in the fresh stool.	Movements sluggish.
Nucleus more or less distinct.	Nucleus distinct.
Chromatin of nucleus scanty.	Chromatin of nucleus abundant.

ENCYSTED STAGE.

Entamœba histolytica.	*Entamœba coli.*
Cyst smaller.	Cyst larger.
Cyst less refractive.	Cyst more refractive.
Cyst usually contains elongated refractive bodies, "chromidial bodies."	Cysts do not contain "chromidial bodies."
Nuclei never more than 4.	Nuclei 8 or more.
Cyst wall thinner.	Cyst wall thicker.

Trichomonas Intestinalis (Syn.: T. hominis).—This flagellate has a pear-shaped body, measuring 3 to 4μ in breadth and 10 to 15μ in length. The posterior end terminates in a point. An undulating membrane terminates in a posterior flagellum, and three longer flagella projecting from the anterior end. A cytostome may be seen near the nucleus (Fig. 88). The organism lives in any portion of the intestinal tract in which the contents have an alkaline reaction. The pathogenic role of this parasite is disputed.

T. vaginalis is the term applied to an apparently identical form which is seen in the vagina and occasionally in the urine. The present opinion is that T. intestinalis and T. vaginalis are the same, though it is admitted that the form seen in the urine and vagina is larger and not so definitely pear-shaped.

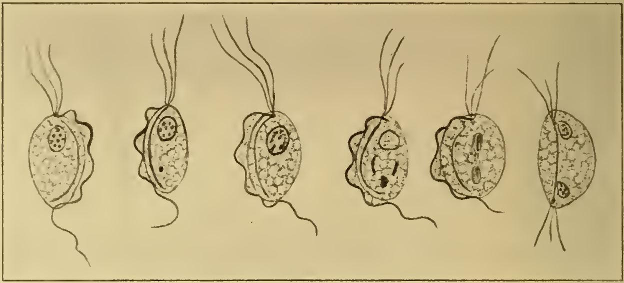


Fig. 88.—Trichomonas intestinalis. (Brumpt.)

Lamblia Intestinalis (Syns.: Cercomonas intestinalis, Megastoma entericum, Lamblia duodenalis).—This bilaterally symmetrical and pear-shaped organism is 10 to 21μ in length and from 5 to 12μ in width. A striking point is the anterior oblique depression which serves as a sucking disc by which the parasite attaches itself to the intestinal epithelial cells. The organism is covered by a thin cuticle (Fig. 89). There are four pairs of flagella, the median pairs being the more active. The nucleus, which is situated in the thinner, anterior portion, is at first dumb-bell in shape but later separates to form two nuclei, one on each side. Two long longitudinal ridges extend from the sucking disc to the posterior extremity. In the flagellated form the parasite is found in the jejunum and duodenum. As it passes into the

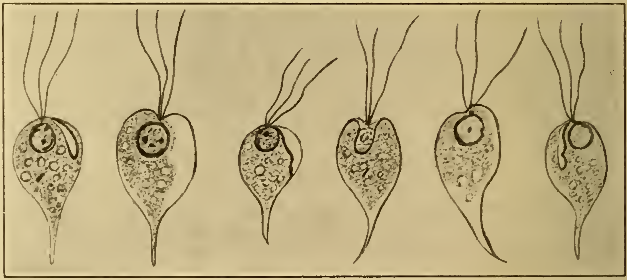


Fig. 89.—Cercomonas intestinalis. (Brumpt.)

larger intestine, it becomes encysted. Oval cystic forms with thick cyst-wall are seen in the stools. L. intestinalis is found associated with many cases of severe diarrhea, and is thought to be the causative agent.

Balantidium Coli (Syn.: Paramecium coli).—The body of this ciliate is oval, measuring from 50 to 70μ in breadth and from 60 to 100μ in length. The cilia are especially marked about the peristome, which is situated at the anterior end. Ectoplasm and endoplasm are separated, the former forming a longitudinally striated cuticle and the latter containing granules of starch, bacteria, drops of fat and mucus, and occasionally red and white blood corpuscles. At the posterior extremity there is an anal opening (cytopyge). A macronucleus and a micronucleus are seen (Fig. 90). Propagation is by transverse division. Encystment takes place under unfavorable conditions. Reports of balantidium infection indicate a fairly wide geographical distribution. Cases have been observed in America. Walker has reported an extensive study from the Philippines. These ciliates are parasitic for hogs and other animals as well as for man. In man, they are evidently the cause of a severe and occasionally fatal diarrhea.

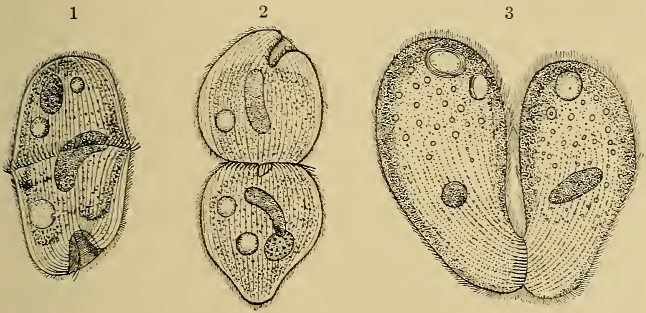


FIG. 90.—Balantidium coli: *1*, *2*, division; *3*, conjugation. (After Leuckart, from Döflein.)

Balantidium Minutum.—This parasite has also been reported parasitic for man. It measures about 20μ long by 14 to 20μ broad. The peristome extends to the center of the body and resembles a fissure.

Trematodes or Flukes.—The trematode worms or flukes are non-segmented, flat, leaf-shaped parasites.

Fasciola Hepatica.—There are two suckers, one at the anterior end, the oral aperature, and one adjacent to it on the ventral surface, the ventral sucker. The dimensions are, length about 20 to 30 mm., breadth 8 to 13 mm. Spines are seen on both dorsal and ventral surfaces. It is a hermaphrodite. The ovary is near the anterior end and the testes, which ramify greatly, occupy a large portion of the body. The uterine rosette is in the anterior portion of the body. The intestine bifurcates and ramifies. The eggs are oval, yellowish-brown in color, are provided with lids (opercula) and resemble the ova of Dibothriocephalus latus, except that they are much larger, averaging about 70 to 132μ.

The liver fluke inhabits the biliary passages of herbivorous animals and is of economic importance on account of the fact that it infects sheep and produces what is termed "liver-rot," thereby causing great loss. Only occasional cases have been reported in man and in most of these the infection has been rather mild, having been discovered in some instances only at autopsy. Diagnosis can be made during life only by finding the ova in the stools. The parasite is found in Europe, and has been reported in North and South America, North Africa, Australia and Asia. The intermediary host in which development of the larva (miracidium) takes place, is the snail.

Opisthorchis Felineus (Syns.: Distoma conus, Distoma lanceolatum, Distoma sibiricum).—The cat-fluke usually measures from 1.5 to 2 mm. by 8 to 11 mm. and is yellowish-red in the fresh condition. The flat body shows a conical neck. There are two suckers. The posterior end is either rounded or pointed. The oval eggs, measuring 11 by 30μ, show a clearly defined lid at the pointed pole. This species inhibits the biliary ducts and gall-bladder of the domestic cat, and is also found in the dog, fox, and in other animals. It is found in man, where extensive changes may take place in the liver. Numerous cases have been reported from Siberia. The intermediate host is in certain fish and the infection is presumably acquired by eating uncooked fish.

Clonorchis Endemicus (Syn.: Distoma japonicum).—Within this genus are probably two species, C. endemicus and C. sinensis. The differentiation has not been clearly made, however, and the existence of two species is denied by some. The genus Clonorchis comprises the more important human liver-flukes. In shape the flukes resemble the opisthorchis felineus, but have been separated because the testicles branch while those of Opisthorchis are lobed. The fluke is common in Japan and China and is diagnosed by the presence of eggs in the stools. The ova measure about 13 to 16μ by 26μ, and show sharply defined lids. The exact mode of infection is not definitely known, but it is felt that a mollusc is the primary intermediate host and a fish the secondary intermediate host.

Fasciolopsis Buski (Syn.: Distoma buski).—This thick, brown fluke has a breadth of from 5.5 to 14 mm. and a length ranging from 24 to 70 mm. The ovary and shell-gland are in the center of the body with the testes behind and the uterus in front. The ventral sucker is three or four times the size of the oral sucker. The eggs measure from 77 to 88μ in breadth and from 120 to 140μ in length. They are thin-shelled and show a small lid. F. buski lives in the intestine of pigs, occasionally in man. In man, it is found in Siam, India, Assam, Sumatra, and China, and is particularly common in Cochin China. The larval stage is said to take place in shrimps.

Genus Schistosomum.—The genus Schistosomum differs from the other flukes in that the sexes are separate. The bodies of the male widen behind the ventral sucker, and the lateral portions roll ventral-wards to form a partially closed canal (canalis gynecophorus), within which the female lies. The female is slender and filiform, about 20 mm. long, while the male is 12 to 14 mm. long. The eggs are oval and somewhat elongated, measuring about 40 to 60μ in breadth by 120 to 150μ in length. They do not have lids, but show either lateral or terminal spines. They are called blood-flukes, since they invade the circulatory system. Infection with the blood-flukes is termed bilharziasis since Bilharz was the first to discover the parasite and describe the disease. The early development of the worm takes place in the liver. The young worms travel from this point in the portal veins and its tributaries against the blood stream. Symptoms frequently noted are due to bladder involvement, such as hematuria and vesical irritation and those due to lodgment of the worms in the hemorrhoidal veins, such as "bleeding piles" and diarrhea. The eggs are found in the urine and in the feces. There are three human species.

Schistosomum Hematobium (Distoma Hematobium Bilharz).—The male shows four or five large testes. The gut stem is short. In the female, the ovary is in the posterior half of the body and the uterus is long and voluminous. The ova show terminal spines, have no lids, and measure 40 to 60μ in breadth and 120 to 150μ in length. The geographical distribution covers many regions in Africa while isolated cases have been reported from Cyprus, Greece, India, and Arabia.

Schistosomum Mansoni.—In this form, the eggs show lateral spines. The male has eight small testes and the gut-stem is very long, while the ovary of the female is in the anterior half of the body and the uterus is short. This species is said to be found only in the Congo, in the West Indies, in Brazil, and in the Southern States of this country.

Schistosomum Japonicum.—This species differs chiefly from the preceding ones in the fact that the skin of the adult worm is not tuberculated. The lower part of the male infolds to a greater extent. There are six to eight irregularly elliptical testes. The eggs have small lateral spines or thickenings, and opposite them cap-like thickenings have been described, though Stitt states that there are no spines. The embryos hatch out in the water in a very short time, and will live there for twenty-four hours. The "larvæ" which are formed penetrate the skin and in some unknown way reach the portal veins. The penetration of the skin is marked by itching of the legs. The embryos also develop in molluscs. This species occurs in China, Japan, and the Philippines. Cattle, dogs, and cats, are infected as well as man. In man, the effects produced are anemia due to loss of blood, ascites, phlebitis and thrombosis, bloody diarrhea, etc. The eggs are readily found in the feces.

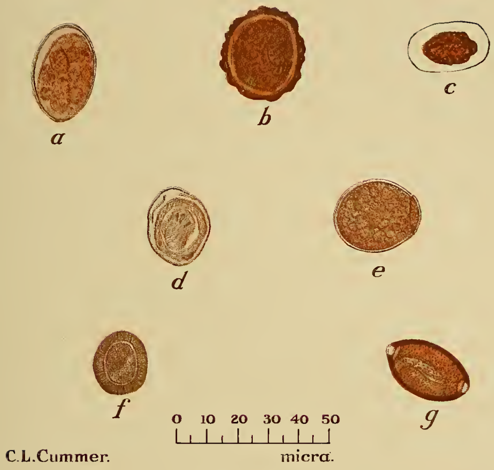
Class Cestoda.—The flat worms are divided in the two classes, Trematoda and Cestoda. The Trematoda, or flukes, have been discussed in the preceding paragraphs. The Cestoda comprise the tapeworms. They have been compared by Stitt on anatomical grounds to a series of individual flukes united in a ribbon-like colony. The adult worm is composed of a scolex or head and of a number of proglottides, or segments. The scolex serves to attach the worm to the host. The head is provided with a sucking apparatus by means of which it fastens itself to the intestinal mucosa. In addition, some species are provided with hooklets. When present, they are carried on the rostellum. The scolex is divided into head and neck. The segments are formed from the scolex, so that the scolex actually produces the colony of which it is the head. The youngest segment is that nearest the scolex and the oldest that at the distal end of the colony. The number of segments varies, T. echinococcus having only three or four and T. saginata having about 2000. The cestoda are hermaphrodites, and each segment contains ovaries, cirrus, uterus, vasa deferentia, genital pores, yolk glands, etc. The proglottides are covered with elastic tissue and absorb their nourishment through the surface.

For the development of the embryo from the eggs formed in the segments, passage through an intermediate host is necessary. The host is different for each species. The eggs leave the host which harbors the adult-worm either free in the feces or contained in a detached segment. Their entry into the intermediate host is purely passive. After ingestion, the embryonic envelope is dissolved in the alimentary tract and the six-hooked embryo or oncosphere is set free. This bores through the gut and encysts itself in the body tissues. Here the hooklets are discarded and a bladder-like structure is formed in which the scolex is inverted. When tissue containing this cyst is eaten by a suitable host, the cyst wall is dissolved and the liberated scolex attaches itself to the intestinal wall and develops a series of proglottides. The embryo of Dibothriocephalus latus, however, does not form a cyst. In most instances, the adult stage is that seen in man, T. echinococcus being an exception. The larval stage takes place in different animals.

From a practical standpoint, two points are worthy of notice. One is that while segments may be detached from the head, the human host is not freed from the infecting organism until the head has been passed, since it has the power of forming a new series of segments. The other is, that in examining stools for ova of tapeworms, it is important to examine for segments also, since ova are rarely found free in the stools but are contained in the intact segments. The ova of Dibothriocephalus latus, however, are almost constantly present in the feces.

Tænia Saginata (Syns.: Tænia mediocanellata).—The length of the "beef tapeworm" ranges from 4 to 10 meters, and has even reached 36 meters, the head is tiny, 1.5 to 2 mm. in diameter and cubical in shape. There are four suckers but no hooks, hence the name, "unarmed tapeworm." The rostellum is represented by a sucker-like organ. The segments are longer than they are broad. Each segment

PLATE VII



Parasite Ova.

a, Oxyuris vermicularis; *b*, Ascaris lumbricoides; *c*, Uncinaria americana; *d*, Hymenolepis na; *e*, Dibothriocephalus latus; *f*, Tænia saginata; *g*, Trichocephalus dispar. (All but *f* wn with camera lucida).

has a single genital pore, which is laterally placed and alternates irregularly. The uterus shows a median trunk with twenty to thirty-five lateral branches, which ramify still further. There are two ovaries in each segment (Fig. 91). The approximately globular ova are about 30 to 40μ long and from 20 to 30μ broad. The inner egg-shell is usually intact and is surrounded by an embryonal shell which is thick and radially striated. Larval development takes place in cattle. In infected cattle, encysted larvæ (Cysticercus bovis) are found more frequently in the tongue and in the jaw-muscles than in other tissues. The cysticercus is rarely found in man. Infection is caused by eating uncooked or underdone meat. Distribution is extremely widespread. T. saginata is the species most frequently encountered in this country.

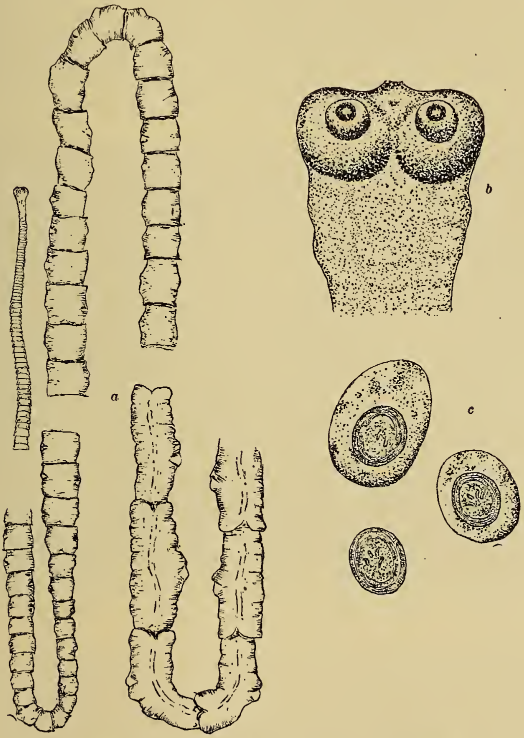


Fig. 91.—Tænia saginata. *a*, natural size; *b*, head, much enlarged; *c*, ova, much enlarged. (Simon.)

Tænia Solium (Syn.: Tænia dentata, Tænia humana armata).—The average length of the "pork tapeworm" is from 2 to 3 meters though it may be longer. The globular head is very small, 0.6 to 1.0 mm. in diameter. A short rostellum is armed with a double row of hooks, about 22 to 32 in number. There are four suckers, which are not as powerful as those of T. saginata. The segments increase in size gradually. Those shortly posterior to the head are almost square while those mature enough for detachment are longer than they are broad. The genital pores are on the side and alternate with considerable regularity. In contrast to T. saginata, the median trunk of the uterus has only seven to ten lateral branches on a side which in turn show tree-like branching. T. solium has three ovaries (Fig. 92). The eggs are quite similar to those of T. saginata and cannot be distinguished on microscopic examination. The fully developed adult form is found only in man. The larval stage (Cysticercus cellulosæ) is found in the domestic pig commonly, and occasionally in some other animals, such as the dog, sheep, monkey, etc. The larval stage has been found also in man. T. solium, not often found on this continent, is much more frequent in Europe.

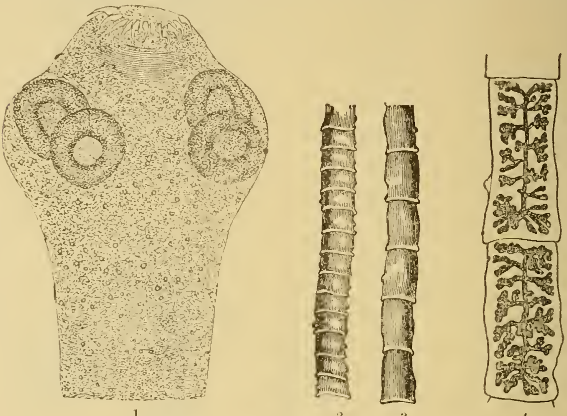


FIG. 92.—*1*, head of Tænia solium; magnification, 50; *2*, *3*, mature and semi-mature segments, natural size; *4*, two proglottides with uterus, twice magnified. (From Ziegler, after Leuckart.)

Tænia Echinococcus.—In contrast to the two species of tænia just described, the T. echinococcus passes the adult stage of its development in the intestinal tract of a lower animal (dog) and the larval stage in man. It is described here not because the ova are found in the feces, since they are not, but simply to complete the description of the genus.

Indeed, diagnosis is usually made only by finding the cysts on the operating table or at postmortem.

The adult worm is one of the smallest of the tapeworms. There are only three or four segments and the total length is about 6 mm. The head shows four suckers and a double row of hooklets. The larval stage is found in hogs and sheep as well as in man. After the ova have been ingested, the embryo leaves its shell and bores through the walls of the alimentary tract, lodging in the liver or other organ where the cyst is formed. This is at first merely an envelope made up of a laminated wall enclosed granular fluid. Later it is seen that the wall has two layers. From the inner or germinal layer are developed brood capsules and scolices. In the enclosed brood capsules, the germinal layer is external. Scolices may be formed either on the inner or outer wall of the brood capsules. The original cyst is termed the mother cyst, the brood capsules forming daughter cysts. Ordinarily all of these contain scolices. The multiple cysts which are formed are termed hydatids. In examining cysts found at operation or autopsy, search should be made for fragments of laminated hyaline membrane and for hooklets. The latter are shed by the scolices. Man is infected by association with dogs. Hydatid disease is rather uncommon in this country but is frequent in Iceland, Central Europe, the Scandanavian peninsula, Australia, Paraguay and Argentine.

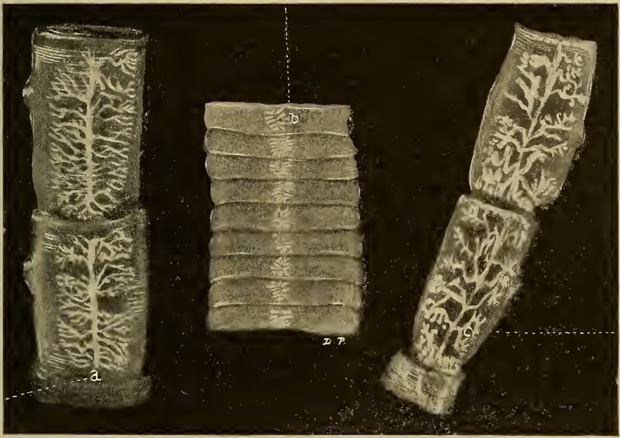


Fig. 93.—Segments of tapeworm: *a*, Tænia saginata; *b*, Bothriocephalus latus; *c*, Tænia solium. (Simon.)

Hymenolepis Nana (Syn.: Tænia nana).—This is a small tapeworm, 10 to 45 mm. in length (Fig. 94). The hooklets, about 24 to 30 in number, are arranged in a single row. The segments are short and broad. The laterally placed genital openings, which cannot be seen by ordinary means, are on the same side throughout. The distal segments are entirely filled with eggs and disintegrate spontaneously.

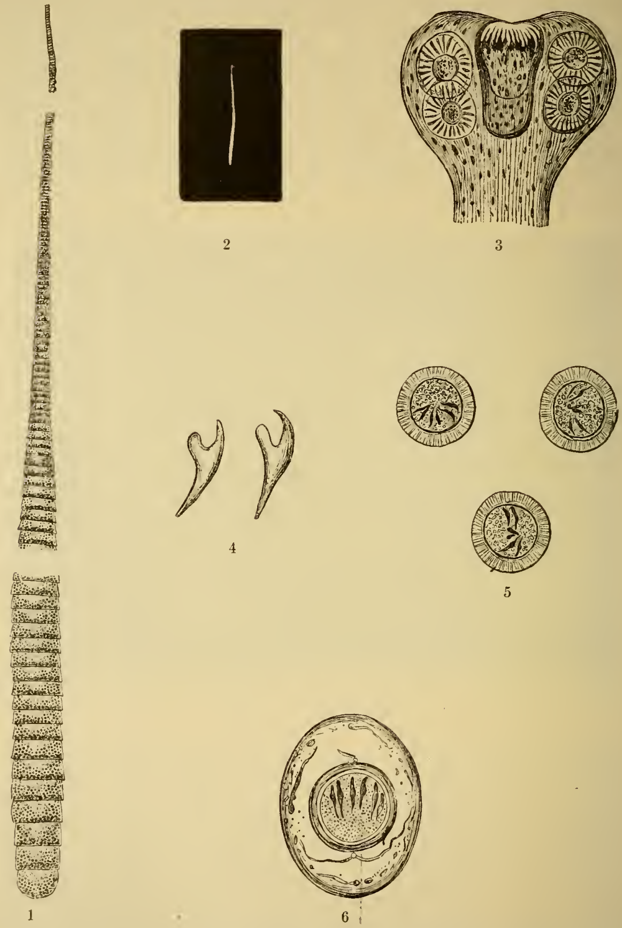


FIG. 94.—Hymenolepis nana: *1*, body; *2*, natural size; *3*, head; *4*, hooklets; *5*, eggs; *6*, egg magnified 600 times. (Mosler.)

It is stated by Stitt that after tænicide treatment the entire worm is usually secured in an advanced stage of decomposition.

The ova are oval or globular in appearance, 30 to 48μ across. There are two coats separated by intervening fluid substance. At either end of the inner coat is a projection from which proceed the filamentous bodies seen between the two coats. The intermediate stage of development is in the rat. The exact details are not known. The cystic stage (cercocyst) has been seen in man. It is thought that this may continue its development in the human host and give rise to a number of adult forms, thus accounting for the cases of heavy multiple infection. Cases are numerous in Sicily and in recent years many have been reported in the United States. In fact, this tapeworm is regarded by some as the most frequent in the United States. It may cause distinct symptoms, including loss of appetite and nervous disorders.

Hymenolepis Diminuta (Syn.: Tænia diminuta).—The small tapeworm is common in rats and there are a limited number of cases on record of human infection. It measures 20 to 60 mm. in length. The segments are numerous, 600 to 2000 in number. The club-shaped head is small and is not armed with hooks. The round or oval eggs have a thick-yellowish outer coat showing radial striping, a thin, double, inner embryonal shell, and a middle granular layer between the two shells. The intermediate host is the rat flea. Human infection has been reported both on the American continent and in Europe.

TABLE SHOWING CHIEF CHARACTERISTICS OF THE MORE COMMON TAPEWORMS.

		Dibothriocephalus latus.	Tænia solium.	Tænia saginata.	Hymenolepis nana.
Length		2 to 9 meters	2 to 3 meters	4 to 10 meters	10 to 45 mm.
Head		Almond shaped, 2 to 3 mm. long	Globular, 0.6 to 1 mm. diam.	Cubical, 1.5 to 2 mm. diam.	Globular, 0.25 to 3 mm. diam.
Suckers		Two grooves only	Yes	Yes	Yes.
Hooks		None	2 rows	None	20 to 24, in 2 rows.
Number of segments		3000 to 4000	800 to 900	More than 1000	Up to 200.
Shape of segments .		Usually broader than long	Longer than broad	Longer than broad	Broader than long.
Genital opening .		Middle of segment	Alternately lateral, first on one side, then on other	Irregularly alternate	Lateral, all on same side.
Uterus		Simple convolutions; near center	Median tube with lateral branchings	20 to 25 branches, breaking up into smaller tubes	
Ova	Size . . .	45 by 68–71 μ	20 to 30 by 30 to 40μ	Same as T. solium	30 to 48 μ.
	Shell . . .	Thin; refractile	Thick, striated	Same as T. solium	2 coats and intervening fluid substance.
	Lid . . .	Yes, at pole	None	None	None.
Intermediate host .		Fish	Pig (dog, sheep, monkey, etc.)	Beef	Rat.
Possible symptoms .		Severe anemia	. . . .	. . . .	Nervous symptom.

Dipylidium Caninum (Syn.: Tænia canina).—The adult worm is from 15 to 35 cm. long. The scolex has three or four rings of hooks. The mature segments are longer than they are wide, and are of reddish color. The genital pores lie symmetrically on the lateral borders.

The eggs are globular, 43 to 50μ, and have a thin internal shell, outside which is an albuminous coating, and external to both, a thin vitelline envelope. The parasite is common in dogs and is also found in cats. The intermediate host is the lice or fleas of the dog. Human infection occurs but is not especially common and is seen more frequently in young children than in adults.

Dibothriocephalus Latus (Syn.: Bothriocephalus latus, Tænia lata, Bothriocephalus balticus, Bothriocephalus latissimus).—The "broad Russian tapeworm" has a length ranging from 2 to 9 meters or even longer. The head is almond shaped, and is about 2 to 3 mm. long. There are neither hooklets nor rostellum, the sucking apparatus consisting of two longitudinal grooves on either side of the head. The segments are extremely broad (Fig. 95). The uterus is rosette shaped and the genital pore is on the ventral side. The eggs are deposited in the intestine of the host and passed with the feces. They are rather large, 45 by about 70μ, and brown. At one pole is a small lid. The embryo is ciliated, swims about in the water, and so enters the intermediate host. The intermediate hosts are various fresh-water fish. Infection takes place by eating raw or imperfectly cooked infected fish, notably pike and perch. Human infection with the broad tapeworm is infrequently seen in this country, and when found is usually in an immigrant. It is found in Russia, Sweden, Poland, the coast districts of Germany, Switzerland, Turkestan and Japan, as well as in parts of France. It is of clinical importance on account of the resulting anemia which is often very severe and resembles pernicious anemia on clinical and laboratory grounds.

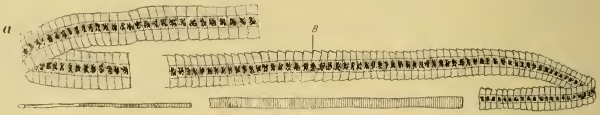


Fig. 95.—Bothriocephalus latus: *a*, *b*, twin segments. (Wilson.)

Class Nematoda.—The nematodes (round worms) are bilaterally symmetrical filiform animals without limbs, usually round and elongated, and covered with a cuticle. The sexes are almost always separate. The male can usually be distinguished because of its smaller size and the spiral or incurved shape of the straight posterior extremity. In both sexes there is an excretory pore which is situated ventrally in the median line near the head. The genital opening of the female is near the midpoint in the median line while that of the male is near the anus. Most of the nematodes develop in damp earth or in water. Few of the nematodes are viviparous.

Ascaris Lumbricoides. In the fresh condition the body is spindle-shaped and reddish-yellow or grayish-yellow in color (Fig. 96). The oral papilla is surrounded with fine teeth. The male is from 15 to 25 cm. long and about 3 mm. in diameter. The posterior extremity is curved and the orifice of the cloaca is surrounded by numerous papillæ. The testicular tube is visible through the body covering and is much folded. The female is from 20 to 40 cm. long and about 5 mm. in diameter. Its posterior end is straight. The vulva is at the junction of the anterior and median portions of the body. The ovaries are convoluted. The ova are elliptical in shape and are covered with a thick, shaggy coat, so that when the egg is seen under the microscope it appears to be covered with knobs or protuberances. Inside of this is a thick, transparent shell. The egg-cell itself is unsegmented and the nucleus cannot be seen because of the coarse granules. When the ova are found in the feces, they show a brown coloration of the outer coat due to staining with bile pigment.

A. lumbricoides is almost universally found, but is more common in warmer climates. It usually infects children. Only a few worms may be present, though large numbers have been reported. Normally, the habitat is the small intestine, though the worms may migrate from here to the stomach (and so be vomited up), and to other parts of the body, where they may be the cause of "worm-abscesses." They have even gained access to the genito-urinary tract and have been passed in the urine. The eggs develop in moist soil but do not leave the shell until taken into the intestinal tract. Infection is by swallowing infected soil.

Belascaris Cati (Syns.: Belascaris mystax; Ascaris mystax).—This belongs to the family of Ascaridæ. The worm is much smaller than the Ascaris lumbricoides, the male being 3 to 6 cm. long and the female 4 to 10 cm. long. From the anterior end of the

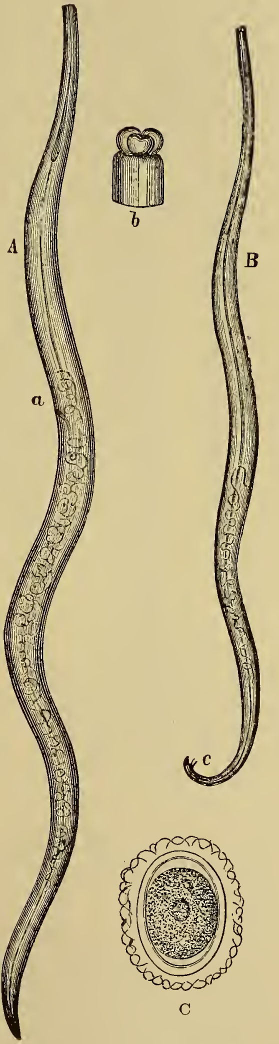


Fig. 96.—Ascaris lumbricoides: *A*, female; *B*, male; *C*, egg. At *a*, the female genital opening; *b*, the enlarged cephalic extremity, with its three lips; *c*, the male spicules. (After Perlo, from Ziegler.)

parasites are wing-like, bilateral projections, which give the parasite an arrow-like appearance. The eggs are 65 to 75μ in diameter with finely honey-combed surfaces.—The host is the domestic cat and dog, rarely man.

Oxyuris Vermicularis.—These worms are popularly known as "pin-worms," "seat-worms," or "thread-worms" (Figs. 97, 98 and 99). They are white and small, the males measuring 3 to 5 mm. in length and the female 10 mm. in length. The tail of the males is incurved. The striated cuticle at the anterior end forms a bulbous-like projection. The eggs are symmetrical and oval, measuring from 50 to 55μ by 16 to 25μ. The shell has a double contour. When the ova are deposited, the embryo within them has already developed.

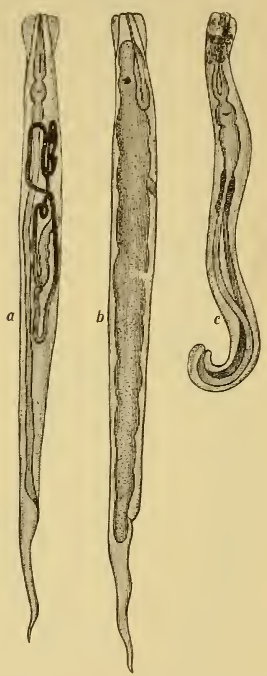


Fig. 97.—Oxyuris vermicularis: *a*, sexually mature female; *b*, female filled with eggs; *c*, male. Magnification, 10. (After Heller, from Ziegler.)

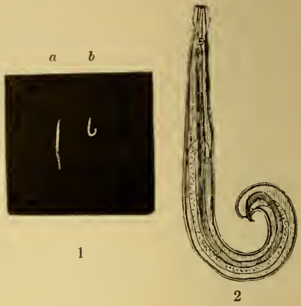


Fig. 98.—*1*, Oxyuris vermicularis: *a*, male; *b*, female; natural size. *2*, magnified. (Simon.)

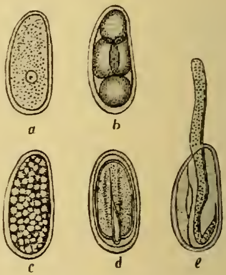


Fig. 99.—Eggs of Oxyuris vermicularis in various stages of development: *a*, *b*, *c*, division of the yolk; *d*, tadpole-like embryo; *e*, worm-shaped embryo. Magnification, 250. (After Zenker and Heller, from Ziegler.)

After ingestion of material containing oxyuris eggs, the adults develop in the intestine where copulation takes place. The male then dies. When the uterus of the female begins to fill with eggs, they leave the cecum and go to the rectum. Here the eggs are laid, inside and outside of the anus. This usually occurs at night and causes intolerable itching. The worms which are hatched may wander to adjoining parts of the body and may be carried under the patient's finger nails to distant parts. An intermediate host is not required for development, so that reinfection of the patient is possible by carrying the eggs under the nails to the mouth. Diagnosis is best made by the examination of the stools after the administration of calomel and salts for the thread-like female adults, which are visible to the naked eye. The worker should be careful not to soil the hands with fecal material, since infection is possible.

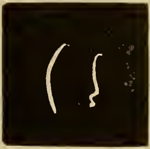


Fig. 100.—Ankylostoma duodenale, male and female. Natural size. (Mosler.)

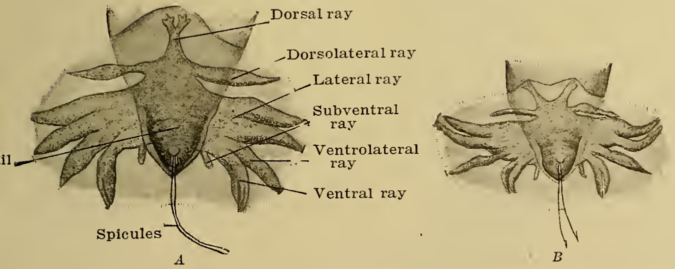


Fig. 101.—*A*, caudal bursa and tail of male Uncinaria duodenalis; *B*, caudal bursa and tail of male Uncinaria americana. Drawn to scale to show difference in size. (After Allen J. Smith.)

Anchylostoma Duodenale.—This is commonly known as the Old World species of hook-worm (Figs. 100, 101 and 102). The worms of both sexes have a mouth capsule which is armed with teeth. There are two pairs on the ventral surface and two protuberances which are really rounded edges of a gap in the edge of the capsule. The cutting apparatus also includes a pair of lancets. The body shows a marked lateral torsion and the head is curved dorsally somewhat. The male measures about

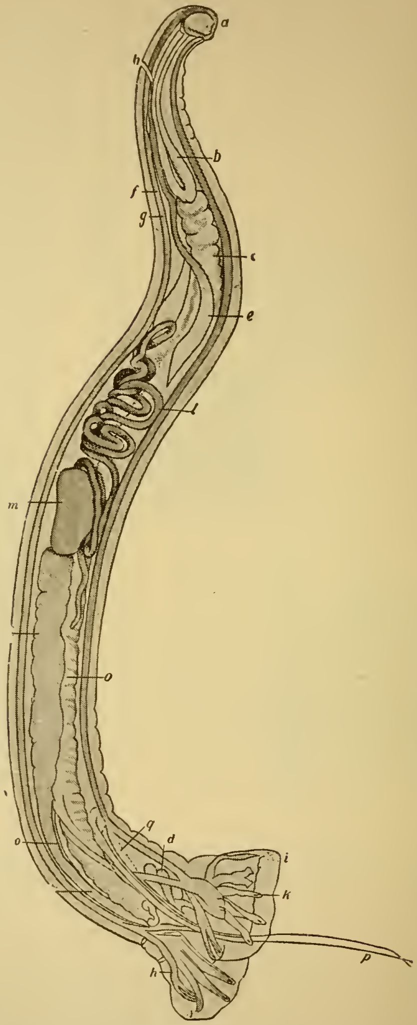


FIG. 102.—Male Ankylostoma duodenale: *a*, head; *b*, esophagus; *c*, gut; *d*, anal glands, *e*, cervical glands; *f*, skin; *g*, muscular layer; *h*, excretory pore; *i*, trilobed bursa; *k*, ribs of bursa; *l*, seminal duct; *m*, vesicula seminalis; *n*, ductus ejaculatorius; *o*, its groove; *p*, penis; *q*, penile sheath. Magnification, 20. (After Schulthess, from Ziegler.)

9 mm. long by 0.45 mm. thick and the female 12 mm. long by 0.6 mm. thick. The male may be distinguished by a wide umbrella-like expansion of the posterior end (copulatory bursa) while the tail of the female is pointed. The vaginal opening of the female opens posterior to the median line. The eggs which are oval with rather broad poles, measure about 34 to 38μ by 56 to 61μ. The contour is single. Between the shell and the granular mass of the ovum is a very striking clear space, which renders the recognition of the egg quite simple. The ovum often shows segmentation into four masses. Embryos are not seen within the eggs in freshly passed stools. The parasites live in the jejunum, less frequently in the duodenum. They attach themselves to the mucosa, and so cause loss of blood. Larvæ enter the body either by the mouth or burrowing through the skin. When the skin is penetrated, there is redness and burning at the point of entry and later swelling of the connective tissue. Through the lymphatics or the blood stream the parasites ultimately reach the intestinal tract. After the eggs have been passed in the stools, the embryos hatch. The embryo (larva) develops under suitable conditions of temperature (best between 25 and 30° C.) and in the presence of oxygen. Man only is infected. An intermediate host is not required. The distribution is fairly wide, including Africa, Egypt, Japan, China, Southern Europe, portions of South America and the Philippines.

The symptoms produced may be very marked, including lassitude, pallor, underdevelopment, loss of weight, anemia of the secondary type of more or less marked degree, the hemoglobin falling as low as 40 or 50 per cent. A differential count usually shows an increase in the percentage of the eosinophiles.

Necator Americanus.—The New World hookworm, first noted in the United States by A. J. Smith and first described by Stiles, shows certain marked points of differentiation from the Old World species. The buccal capsule is small and there are no ventral teeth as in A. duodenale but instead cutting plates, with a dorsal cone which projects into the buccal cavity. Four buccal lancets are seen. The head is bent sharply dorsalwards. The male is 8 mm. long and the female 10 mm. long. The opening of the vulva in the female is in front of the median line. The eggs are somewhat more pointed at the poles than the eggs of A. duodenale, and measure about 36 by 64 to 72μ. The infection is very prevalent in the southern portion of the United States and in Porto Rico.

Strongyloides Intestinalis.—The life-history of this parasite includes two generations, a parasitical or intestinal form, and a free-living form. The former is sometimes referred to as S. intestinalis and the latter as S. stercoralis. The parasitic generation is represented only by the female. In the opinion of most workers reproduction is by parthenogenesis, although some believe that the parasite is hermaphroditic and that the testes degenerate after functioning. The parasite is colorless and *in situ* can scarcely be seen even with a hand lens, so that it is necessary to scrape off the mucosa and to examine the scrapings microscopically. The worm is only about 2.2 mm. in length and from 34 to 70μ in breadth (Fig. 103). The mouth is surrounded by four lips. The anus opens shortly in front of the posterior extremity. The vulva is situated at the junction of the middle and posterior thirds of the body. The eggs resemble the eggs of Anchylostoma duodenalis in appearance. They are about 30 to 34μ across and from 50 to 54μ long and lie in a chain. The eggs are laid in the mucosa of the host and are rarely seen in the stools except after active purgation. The stools, however, may show actively motile embryos which have pointed tails and at first measure about 250μ in length, growing to a length of 500μ. When the temperature is low, the rhabditiform embryos develop into filariaform embryos, which are infective when ingested. At a higher temperature the parasites become sexually mature, copulate, and form the free-living generation.

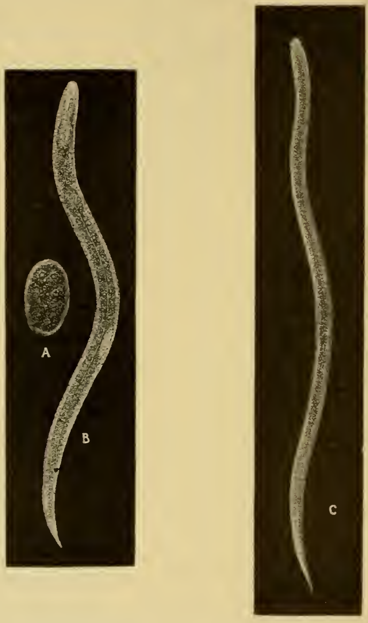


FIG. 103.—*A*, egg of Strongyloides intestinalis (parasitic mother worm); *B*, rhabditiform embryo; *C*, filariaform embryo, derived by direct transformation from a rhabditiform embryo. (Thayer.)

The free-living form has a smooth cylindrical body, pointed at the posterior end. The mouth shows four indistinct lips. The males measure 0.035 mm. in breadth and 0.7 mm. in length. Their posterior end is curved. The females have a straight tail and are 0.05 mm. broad by 1 mm. long. After the eggs have emerged from the shell, rhabditiform larvæ develop with all the characteristics of the parents. When sufficiently grown, they moult and assume the characteristics of the grandparents (parasitic generation). When ingested, they become parasitic; otherwise death takes place.

The parasite was first discovered in so-called Cochin China diarrhea. It is found in Brazil, Africa, and Europe, and cases have been reported in the United States. Infection of man may take place not only by direct entry into the stomach but through the skin.

Trichinella Spiralis (Syn.: Trichina spiralis).—The adult male worm measures about 0.04 mm. in diameter by 1.4 to 1.6 mm. in length. The cloaca is at the posterior end and lies between two caudal appendages which serve to hold the female during copulation. The cloaca is evertible and forms a penis. The female is 0.06 mm. in diameter and from 3 to 4 mm. long. The anus is terminal and gives off embryos (not ova) from the vulva, which is near the anterior end.

In the adult stage, the parasite inhabits the small intestine of man, pig, rat, and wild boar. The adult males die shortly after copulation. The young remain in the body of the host, are deposited by the female in the lymph spaces and carried by the lymph stream to the heart. In the early stage of infection, they may be found in the blood by centrifugalizing a small quantity which has been laked with ten times its volume of dilute acetic acid. Though they are distributed to various parts of the body, the larvæ live only in the striated muscle, where they encyst themselves. The cysts, which are surrounded by a chitinous capsule, measure about 250 by 450μ. The larva are seen curled up in the cysts. They measure about 6μ in diameter by 90 to 100μ in length. They may be found in any of the striated muscles, especially in the tongue muscles and the diaphragm. Diagnosis may be made by excision of a bit of the deltoid or biceps muscles near the insertion and by teasing out the tissue under the microscope to demonstrate the encysted larvæ. A marked eosinophilia is present, sometimes running as high as 50 per cent.

Man is infected by eating infected and insufficiently cooked pork. Pigs are infected by eating rats, who in turn are infected by eating pork about slaughter houses. Since they are cannabalistic, rats continue the infection indefinitely. During the period of invasion, there are marked gastro-intestinal symptoms, vomiting and diarrhea. There may be slight edema. In the second week, myositis is prominent. Mastication, speech, and respiration may be difficult. Eosinophilia is found. The temperature may be elevated to 104 or 105° F. In the third week, encystment takes place. Edema of the face is pronounced. There may be delirium and somnolence. Death may result or the milder cases may progress to recovery.

Trichuris Trichiura (Syn.: Trichocephalus trichiurus, Trichocephalus dispar).—This parasite, commonly known as the "whipworm," lives in the cecum of man, occasionally in the appendix, colon, or even small intestine (Figs. 104 and 105). The male measures 40 to 45μ in length, the female 45 to 50μ. In both sexes, the anterior portion of the worm is slender and whip-like. The ova are striking and characteristic, being barrel-shaped and dark-brown in color, with a perforation at each pole which is closed with a plug of light color.

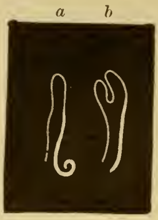


Fig. 104.—Trichocephalus dispar. *a*, male; *b*, female. (Mosler.)

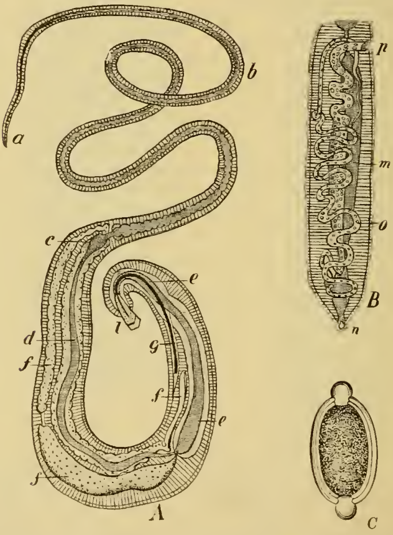


Fig. 105.—Trichocephalus dispar. *A*, male; *B*, posterior extremity of female; *C*, ovum. *a*, head; *b*, cephalic extremity of body with esophagus; *c*, stomach; *d*, gut; *e*, cloaca; *f*, seminal canal; *g*, penis; *l*, bell-shaped penile sheath, with tip of penis; *m*, gut of female; *n*, anus; *o*, uterus; *p*, vaginal cleft. Magnification, 10. (After Küchenmeister and Zürn, from Ziegler.)

They measure about 23μ in breadth and from 50 to 54μ in length. The eggs develop in water or moist soil. Infection in man is common. There is considerable question as to whether any symptoms result.

PRESERVATION AND STAINING OF FLUKES AND CESTODES.

Fixation of Flukes.—The flukes should be placed in a small bottle or test-tube about a quarter full of normal saline solution. The contents are shaken well to extend the worm and an equal bulk of a saturated solution of mercuric chloride (10 gm. to 160 cc. of water) is added. This is shaken vigorously for a few moments, when the worm is transferred to 70 per cent. alcohol.

For large flukes, the worm should be flattened out between two glass slides held together by rubber bands, and fixed by immersion in a saturated solution of mercuric chloride.

Staining may be accomplished after fixation. The mercuric chloride should be removed with tincture of iodine. Then the parasite is allowed to stand overnight in a *dilute* solution of carmine. Differentiation may be accomplished by allowing the preparation to remain in acid alcohol from one to twenty-four hours. (Acid alcohol may be prepared for this purpose by adding 5 drops of HCl to 100 cc. of 70 per cent. alcohol.) The specimen should then be dehydrated by placing it in increasing strengths of alcohol, when it may be cleared in phenol (phenol, 4 parts, water 6 parts). It may then be transferred to 95 per cent. alcohol, next to xylol, and finally to balsam; or it may be mounted in glycerine, according to the method for ova given below.

Fixation of Cestodes.—The worm is placed in a saturated solution of mercuric chloride to which acetic acid has been added to the extent of 1 per cent. Plenty of fixation should be used. The fixative should be warmed to 70 or 80° C. and allowed to act for fifteen minutes. The worm may then be transferred to 70 per cent. alcohol. The mercuric chloride should be removed by treatment with tincture of iodine. Staining, dehydration, and mounting may be accomplished as directed for the flukes.

Preservation of Ova in Feces.—Seventy per cent. alcohol is heated in a beaker to a temperature of about 70° C. The feces are added, about one part of feces for every 9 parts of alcohol. The mixture is stirred and then allowed to settle. The sediment may be transferred to a bottle of fresh 70 per cent. alcohol.

The ova may be kept in a glycerine jelly. Three strengths of glycerine are prepared in 70 per cent. alcohol, 5, 10 and 20 per cent. The alcohol is poured off the sedimented feces, and is replaced with 5 per cent. glycerine. After standing for an hour or two, this is in turn poured off and replaced with 10 per cent. glycerine, which is in turn replaced with 20 per cent. glycerine. The latter is allowed to stand, exposed to the air but protected from dust, so that the water and alcohol will evaporate. A few drops of glycerine are added from time to time so that the ova are finally in pure glycerine.

CHAPTER V.

THE EXAMINATION OF SPUTUM.

Precautions Against Infection.—The specimen should be handled with care. Spread a newspaper on the work-table and perform all steps on it. When through, fold the paper up and burn it or have it autoclaved. A basin containing 1 : 20 phenol solution should be at hand. When the examination is completed, put into it corks, extra slides, bottle, Petri dishes, or any other glass-ware which has come into contact with the sputum. This should then be sterilized either in the autoclave or by boiling. If particles of sputum are dropped on bench or floor, flood with 1 : 20 phenol or 1 : 1000 mercuric chloride at once.

Obtaining the Specimen.—When a patient is asked to furnish a specimen of sputum he should be told that the sputum desired is that which he coughs from "deep down" (from the lungs) and not that which comes from the mouth, nose or throat. In office or dispensary practice the patient should be furnished with a clean, dry, wide-mouth bottle.

Small children usually swallow sputum. Sputum may be obtained from them by irritating the pharynx with a cotton-wrapped swab or with the finger, wrapped with a piece of sterile gauze. The sputum raised by the resulting cough can be caught on the swab or gauze. Another method is to obtain the stomach contents mixed with sputum by passing a small catheter.

Method of Procedure.—The sputum should be subjected to careful macroscopic as well as microscopic examination. The following serves as an outline of the points which should be covered on a routine examination:

(*a*) *Macroscopic Examination.*—Amount; consistency; odor; color; presence of fibrinous casts, Curschmann's spirals, Dittrich's plugs, bits of lung tissue or cartilaginous rings, parasites, concretions or foreign bodies, ray fungus particles.

(*b*) *Microscopic Examination—Unstained Specimen.*—Leukocytes; epithelial cells; red blood cells; heart failure cells; elastic fibers; Curschmann's spirals; moulds; parasites; crystals. *Stained Specimens.*—Stain with Gram's stain; stain for tubercle bacillus; note the types of epithelial cells and leukocytes.

(*c*) *Special Examinations.*—When indicated, specimens may be stained with special stains to demonstrate other microörganisms and eosinophilic leukocytes.

ORDER OF PROCEDURE.

The gross appearance of the sputum should be noted. Prepare slides and stain for tubercle bacilli. Make a slide by the "impression method" and stain with Gram's stain. While decolorization is going on, examine microscopically a particle of unstained sputum. Special stains, as Wright's stain to demonstrate eosinophilia, stains for influenza bacillus and for capsules, etc., should be made when the clinical or the preliminary findings with Gram's stain point to their desirability.

GROSS APPEARANCES OF THE SPUTUM.

While the gross characteristics of the sputum should be studied and noted carefully when a report is made, the laboratory worker should not allow himself to be biased by the appearances, nor should he allow the apparently normal gross appearance of the sputum to deter him from making a careful microscopic bacteriological examination.

Description.—In describing the sputum, use is usually made of the terms serous, mucous, mucopurulent, or bloody. These are self-explanatory. The macroscopic description should be completed with notation of the following characteristics:

Amount.—This can usually be observed only in those who are confined to their beds. The statements of the ambulatory patients regarding the amount of the expectoration are often unintentionally misleading. The amount is greatly increased in edema of the lungs, in bronchorrhea, acute bronchitis (especially with cavity formation), in pulmonary tuberculosis with cavity formation, in abscess or gangrene of the lungs, and after the perforation of an empyema into the pulmonary tissue.

Consistency.—The sputum is of thin, watery consistence in edema of the lungs and occasionally in abscesses, gangrene, and large tuberculous cavities. It is especially thick and tenacious in bronchial asthma, the earlier stages of pneumonia, and occasionally in catarrhal conditions.

Color.—Pale yellow or green sputum is occasionally seen in acute bronchitis. The sputum in pulmonary edema has a bright pink tinge. Red coloration is usually due to the presence of blood. A bright-red color is seen when a small vessel has been ruptured and occasionally in pulmonary infarction.

A brownish-red color is due to changed blood and may be seen in pulmonary gangrene. Dark-brown, "prune-juice" colored sputum is expectorated in certain stages of pneumonia. A bright-green color, due to the presence of bile pigment, is sometimes seen after the rupture of a liver abscess into the lung. A green color is also seen at times in pneumonia, especially during slow resolution where the blood pigment is changed to hematoidin and this is oxidized to biliverdin. Black sputum is seen in coal-handlers and in those who dwell in regions where much soft coal is burned.

Odor.—Ordinarily the sputum is odorless, though the expectoration from large bronchiectatic cavities and from gangrene of the lung has a particularly offensive odor.

Fibrinous Casts.—Casts of the bronchi, representing a portion of the bronchial tree, may be quite extensive in size (Fig. 106). They may be seen in fibrinous bronchitis, in pneumonia during the stage of consolidation and in diphtheria.

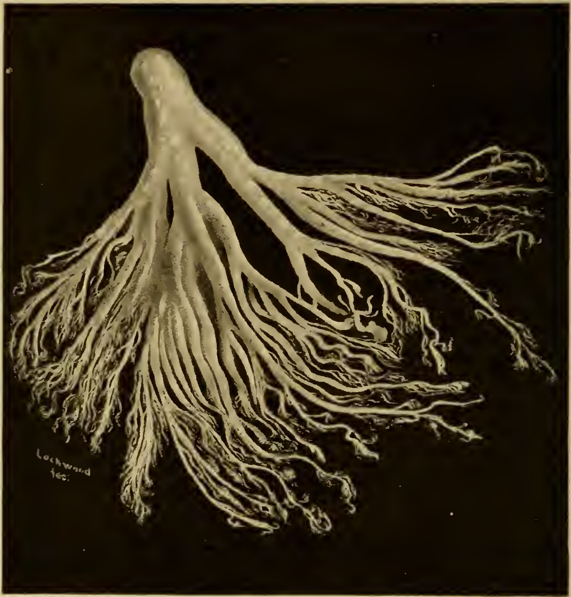


FIG. 106.—Expectorated cast from a case of fibrinous bronchitis. Three-fourths natural size. Drawn from fresh specimen. (After Bettmann.)

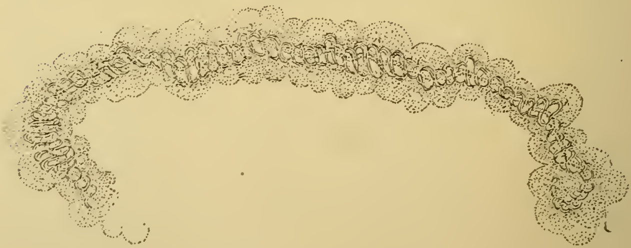


FIG. 107.—A Curschmann spiral from a case of true bronchial asthma (enlarged). (Simon.)

Curschmann's Spirals.—These small, spiral bodies, which are visible macroscopically, may occur in two forms, either as spirally-twisted strands of mucus in which are enclosed leukocytes (particularly eosinophiles) and Charcot-Leyden crystals, or as mucus twisted around a central fiber (Fig. 107). They are seen in cases of bronchial asthma, particularly at the end of the paroxysm, and occasionally in acute bronchitis, acute lobar pneumonia, and chronic pulmonary tuberculosis.

Dittrich's Plugs.—Dittrich's plugs are bodies varying in size from that of a millet seed to that of a bean. They are of yellowish-white color and when crushed give rise to a most disagreeable odor. Microscopically they are found to contain cell detritus, fatty acid crystals, fat drops, and bacteria. They are from the bronchi and are quite similar in appearance to the plugs which are squeezed from the tonsillar crypts.

Bits of Necrotic Tissue.—Bits of necrotic tissue are seen occasionally in cases of gangrene and abscess, and more rarely in pulmonary tuberculosis. Often they are so small that microscopic examination is necessary to determine their true nature. They may be portions of pulmonary tissue or fragments of cartilaginous rings from the cartilages of the traches, bronchi, or larynx.

Concretions.—In most cases, these are calcified tuberculous material.

Parasites.—With echinococcus infection of the lungs, cysts may be expectorated and hooklets have been identified in the sputum. Particles of ray fungus may be seen in the form of sulphur granules.

MICROSCOPIC EXAMINATION.

Unstained Specimen.—Much information may be gained merely from the examination of an unstained specimen. In fact, the "so-called heart-failure" cells or elastic fibers would be overlooked in the examination of the specimens stained by the usual methods. While making this examination, the number of the leukocytes, number and type of epithelial cells, and presence of red blood cells should be noted. After completing the examination, care should be taken to sterilize the slide.

The field should be darkened by dropping the Abbé condenser and by partially closing the diaphragm just beneath the stage. The material should be placed on an object slide, and covered with a cover-slip.

"Heart Failure Cells" (Herzfehlerzellen).—The alveolar endothelial cells are four or five times the size of a leukocyte, oval in outline, with granular cytoplasm and a vesicular, oval nucleus. They are found in greater or less number in all sputa. In cases of chronic passive pulmonary congestion due to cardiac decompensation, they may become partially filled with hemoglobin pigments, which may be in the form of hematoidin crystals, or amorphous masses or scales of hemosiderin. When the pigment is hemosiderin, it will give an iron reaction when treated with a dilute solution 5 per cent. potassium ferrocyanide and a dilute 5 per cent. solution of hydrochloric acid. This reaction may be carried out on the slide by running the reagents under the cover-slip, watching the results under the microscope. The granules turn Prussian-blue. Recognition of these cells may have some importance in completing the picture of bronchitis of cardiac origin. Care should be taken not to mistake for "heart failure cells," alveolar cells which merely contain black particles of carbon.

Curschmann's Spirals.—The macroscopic appearance has been described. On microscopic examination they will be found to consist of spirals in which are enmeshed eosinophilic cells and Charcot-Leyden crystals. Examinations should be made with both low power and high power objectives.

Elastic Fibers.—Elastic fibers may be found in gangrene and abscess of the lung and in pulmonary tuberculosis. Their recognition may be of considerable importance in the diagnosis of early pulmonary tuberculosis. They may be seen when the sputum is pressed between two glass plates in the Sir Andrew Clark method. In making the search, a black background should be employed. Suspicious bodies may be picked up with a teazing needle, placed on a slide and covered with a cover-slip. The fibers will be seen arranged as though about the alveolar structure.

If it is not possible to find them in this way, the sputum may be digested with sodium hydrate which serves to dissolve everything except the more resistant elastic tissue. A portion of the sputum is boiled with approximately an equal quantity of 10 per cent. sodium or potassium hydrate. After the mixture has been allowed to stand overnight, it is centrifugalized and the sediment placed on a slide and examined. If desired, the fibers may be stained with orcein.[1] The supernatant fluid is poured off the sediment, and distilled water is added in order to free the sediment from alkali. The tube is again centrifugalized and the water is poured off. A few drops of the orcein stain are added, when hydrochloric acid is dropped in until a violet color appears. The tube is placed in boiling water for about five minutes, when the contents are decolorized with acid alcohol.[2]

Fibers from food material can be distinguished only by the absence of alveolar formation in their arrangement.

Crystals.—Under occasional circumstances, various forms of crystals may be seen in sputum. The appearance of most of the crystals has been described in the chapters on either Urine or Feces.

Charcot-Leyden crystals are colorless, octahedral in form, dissolving in water and acetic acid and staining with eosin (Fig. 108). They are found in the sputum of cases of bronchial asthma and are often enmeshed in Curschmann's spirals. *Leucin and tyrosin* arise from the decomposition of protein. They are seen in sputum after the rupture of an empyema into the lung and occasionally after the perforation of a liver abscess into the lung. *Fatty acid crystals* are seen in gangrene of the lungs and occasionally in chronic phthisis. *Cholesterol crystals* are found in the sputum of cases with chronic lung abscesses and where there are cavities as in chronic tuberculosis. *Hematoidin crystals* are in the form of rhomboids from whose corners project needles or curved filaments. They are bright yellow in color and dissolve in chloroform. They occur in the sputum rarely, appearing only when extravasated blood has remained in the pulmonary alveoli for a long time. *Hypomycetes* (Molds). Various forms of molds are often found in the sputum as a result of contamination. Certain varieties may be pathogenic. There are hyphæ (jointed rods) and spores. The rods are often branched and are frequently arranged in the form of a network. The columella is the rounded termination of an aërial hypha, which projects upward and carries the fruiting organism bearing the conidia or spores.

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[1] Orcein stain is prepared by dissolving a gram of orcein in 80 cc. of 95 per cent. alcohol, and 35 cc. of distilled water, adding 40 drops of strong hydrochloric acid.

[2] Acid alcohol for this is prepared by adding 1 cc. of strong hydrochloric acid to 200 cc. of 80 per cent. alcohol.

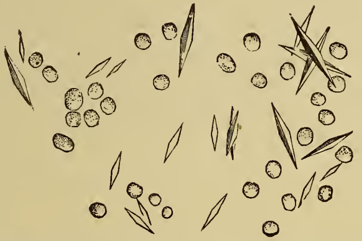


Fig. 108.—Charcot-Leyden crystals. (Scheube.)

An organism, which they have termed *Monilia,* has been described by Boggs and Pincoffs in a fatal case with abscesses in breast and axilla communicating with a cavity between the two layers of the pleura. The sputum was thin, gruel-like, odorless, and faintly tinged with blood and contained small gray masses which showed mycelial threads and yeast-like cells when examined microscopically. The organism could not be distinguished from that described by Ashford in tropical sprue. Both hyphæ and sporulation forms were Gram-negative. Growth on glucose agar was luxuriant.

Blastomycetes (Yeasts).—The blastomycetes reproduce in the tissues by budding or by sporulation only. They may be found in the sputum in pulmonary blastomycosis, (Fig. 109) and are apparently identical with the organisms found in pus and tissues in subcutaneous blastomycosis. The infection is systemic, as shown by the studies of Montgomery and Ormsby and Stober and others. They are seen in the sputum as round or oval bodies with a highly refractile, double contour. They are Gram-positive, stain readily with ordinary stains, and may be recognized microscopically even in unstained specimens. The organism may be cultivated on ordinary media. Growth is seen in from two to fourteen days. In cultures kept at room temperature, aërial

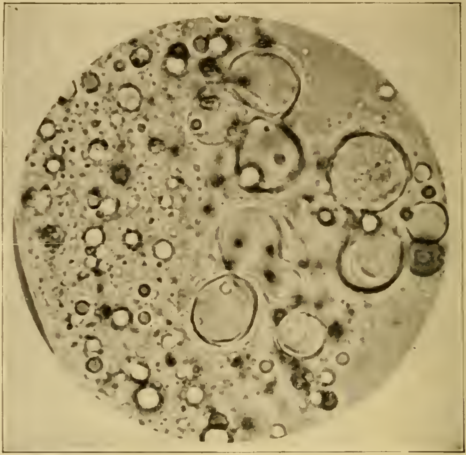


Fig. 109.—Blastomycetes. Smear from sputum mounted in 1 per cent. potassium hydrate solution, showing circular and budding organisms. × 1200. (Eisendrath and Ormsby.)

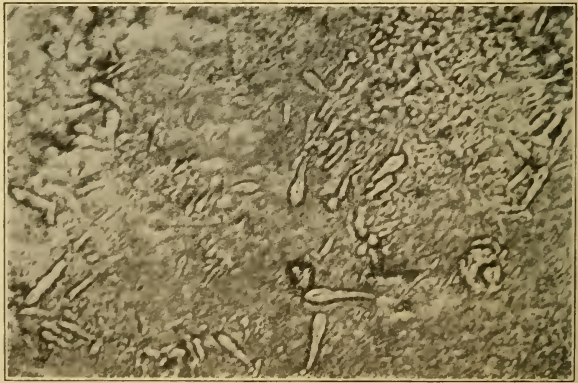


Fig. 110.—Crushed actinomyces granule from fresh sputum. Unstained. × 750. (Lord.)

hyphæ are formed and microscopic examination shows finely branching mycelium with a few small, spore-like bodies, while cultures kept at 37° C. show a moist growth and budding forms.

Actinomycosis.—The characteristic finding in the sputum is the "sulphur granules." Macroscopic recognition is important. When one of the "granules" is pressed out on a slide beneath a cover-slip, it will be seen to consist of a net-work of fine threads, at the ends of which are bulbous-shaped granules (Fig. 110). The central portion may be stained with Gram's stain. The bulbous ends are degenerate structures and stain with eosin though not with Gram's stain. The organism may be cultivated in deep glucose agar stabs since it is essentially an anaërobe.

Parasites.—Attention has been called to the possibility of finding the hooklets from *echinococcus cysts* in the sputum. These may be identified microscopically. *Amebæ* may be found from amebic liver abscesses which have ruptured into the lung. In looking for them, the technic described elsewhere (see page 325) should be followed. *Filarial embryos* and the ova of *Paragonimus westermannii* are seen in the sputum. These ova, which are constantly present in the expectoration of infected individuals, are brownish-yellow and about 49 to 81μ in diameter. The lung-fluke itself may be expectorated. It is plump, oval in shape, and faint reddish-brown in color. It is 3.5 mm. thick, 4 to 6 mm. wide, and 7.5 to 12 mm. long. There are two suckers, the oral one below the anterior termination, the ventral one in front of the middle of the body. The surface is covered with spear-shaped spines. The branched ovary is on one side, the uterus is on the opposite side, and testicles are posterior.

Man and dog are hosts for the adult parasite. The flukes are found in cysts in the lungs. The cyst walls are formed of thickened connective tissue. The larval stage (cercaria) is found in two intermediate hosts, molluscs and fresh-water crabs. When ingested by man, it is thought that the fluke goes through the intestinal wall, perforates the diaphragm, invades the pleural cavity, penetrates the lung tissue and there becomes encysted. The symptoms are those associated with bronchitis and peribronchitis, cough and hemoptysis, so the infection has been called endemic hemoptysis. The distribution of the lung-fluke is in Japan, China, and Korea.

Examination of Stained Specimens.—**Impression Method of Making Preparations.**—In staining for organisms other than tubercle bacilli and especially when a study of the cell morphology is desired, the "impression method" of making slides should be followed. A drop of mucopurulent material is taken up on a platinum wire and touched to the surface of the slide in a number of different places. It should not be smeared over the surface lest the cells be broken up.

For general examination, a slide should be stained with Gram's stain and one with Loeffler's methylene blue or with toluidin blue. When it is desired to determine the presence of eosinophiles, as in bronchial asthma, a slide made by the impression method should be stained with Wright's stain.

Preparing Slides for the Search for Tubercle Bacilli.—The best method to employ in hunting for tubercle bacilli is that of Sir Andrew Clark. Two pieces of glass (preferably plate-glass) are necessary. One of these should be about 14 to 18 inches square and the other about 6 inches square. A portion of the sputum is placed on the upper surface of the larger piece of glass, and the small piece of glass is placed over the sputum, just as a specimen on an object slide is covered with a cover-glass. The upper piece of glass is pressed down rather firmly and is moved along gradually, while the lower piece remains stationary. The specimen is now spread out in an extremely thin layer. Close watch is kept for the tiny cheesy bodies, which are picked up with a teasing needle and placed on an object slide as they emerge at the edge of the smaller piece of glass. After four or five have been obtained, a smear is made on an object slide by placing over it a second slide, which is pressed down and then pulled away so that a film is made on both slides.

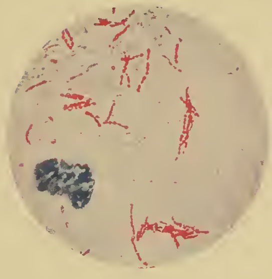
The only objection to this method is the inconvenience of sterilizing the rather unwieldly pieces of glass, especially when many specimens are to be examined. It is more convenient to pour the specimen into Petri dishes. With either method, a dark background should be used. A piece of black paper may be placed under the glass plate or Petri dish. The "rice bodies" or cheesy particles should be looked for with great care. Simple mucus or pure blood or pus should be avoided. Particles of food are often confusing. A teasing needle will be found better suited for picking up the cheesy bits than a platinum loop.

Staining for Tubercle Bacilli.—*Fixation.*—Dry the slides by holding them high over a low flame. Then fix them by passing them quickly through the flame three times, holding the slide with the smeared side down. Care should be taken to avoid overheating or scorching the smears. An unfixed slide may bear infectious material and should be handled accordingly. If it be not stained, it should be passed through the flame several times and thrown in a receptacle containing an antiseptic solution.

Ziehl-Neelsen Method.—After covering the slide with Ziehl-Neelsen carbol-fuchsin solution, hold it over a low flame to steam. The stain must not be allowed to boil or to dry around the edges, since a crystalline deposit is formed which makes a deceptive preparation. Steaming should be continued for at least three minutes, adding fresh stain to replace that lost by evaporation. Many specimens may be stained at the same time by placing them in a beaker or other vessel which is filled with steaming carbol-fuchsin, marking the slides for identification.

The stain is poured off and the film decolorized by immersing in acid alcohol (1 part of strong hydrochloric acid in 99 parts of 85 per cent. alcohol) for a few seconds until almost colorless, when it is placed in

PLATE VIII



Photograph of Human Type of Tubercle Bacilli from Sputum.

Bacilli in red, rest of specimen blue. × 1000 diam. (Fränkel and Pfeiffer).

95 per cent. alcohol until it is evident that no more color is removed. This will require from one to five minutes. The film is counterstained with Loeffler's methylene blue for one minute, washed with water, and dried, and examined with the oil-immersion lens. A mechanical stage is needed for careful search.

The tubercle bacilli appear as bright red, granular or dotted rods since they are "acid-fast" (resisting the decolorization of the acid), while other bacteria, leukocytes, and epithelial cells are blue (Plate VIII). This method of staining is so far superior to Gabbett's method that the technic for the latter will not be given here. While Gabbett's method is somewhat more rapid the results are not as reliable and the appearance of the slide stained with Gabbett's fluid is often far from satisfactory.

Pappenheim's Method.—The slides are stained with carbol-fuchsin as with the previous method. The stain should be poured off without washing and the slide flooded with the Pappenheim's decolorizing fluid. This is poured off slowly, and the slide covered with fresh fluid, which in turn is drained. The process is repeated four or five times. While smegma bacilli may retain the stain after decolorization with the mineral acids and alcohol, they are decolorized by rosolic acid. The method is particularly suited for the examination of urine, where the possibility of contamination with smegma is much greater than in examining sputum.

Much's Granules.—Much described granules which are Gram-positive but not acid-fast. They are regarded as tubercle bacilli because when material containing them has been injected into susceptible animals, tuberculosis has resulted. Since they are found in inactive or quiescent cases the claim has been made that they are less virulent forms. While this may be true, the detection of Much's granules is not generally accepted as a reliable clinical method because of the difficulty of differentiating them from other organisms or granular material. It is recognized that cheesy material contains a wealth of chromatin particles emerging from the leukocytes in all stages of degeneration. While some of Much's granules may be degenerated tubercle bacilli, others doubtless have their origin in degenerated leukocytes.

Antiformin Method of Preparing Sputum for Examination.—When tubercle bacilli are present in small numbers, they may be difficult to demonstrate with the foregoing methods. Numerous ways have been suggested for the digestion and concentration of the sputum. A reagent is required which will dissolve the cells, mucus, and protein material and many of the bacteria, but will not affect the tubercle bacillus. The resulting digested fluid is centrifugalized so as to concentrate the tubercle bacilli in the sediment. Of the various methods, the one employing antiformin is preferable.

Procedure.—Place equal quantities of sputum and antiformin in a small bottle provided with a tight-fitting rubber stopper. All utensils should be thoroughly clean, and only freshly distilled water employed on account of the possibility of introducing acid-fast bacilli in old distilled water or tap water. Shake the mixture thoroughly until of thin consistency and then pour it into a centrifuge tube. After centrifugalization decant the supernatant fluid and fill the tube with sterile distilled water in order to wash the sediment free of alkali. Again centrifugalize, pour off the salt solution, spread the sediment on a slide, dry it, fix with heat, and stain in the usual way for tubercle bacilli. It may be necessary to add a drop of Mayer's egg-albumen to the sediment to make it adhere to the slide.

The writer's personal experience has been that this method affords no better results than a careful search for caseous particles according to Sir Andrew Clark's method.

Cultivation of B. Tuberculosis (*Petroff's Method*).—In attempting to cultivate the tubercle bacillus, Petroff takes advantage of the fact that treatment with 3 per cent. sodium hydrate destroys staphylococci and streptococci, and that gentian violet in the culture medium inhibits the growth of many contaminating organisms, especially the moulds and the hay bacillus.

The sputum should be obtained in as nearly an aseptic manner as is possible, directing the patient to use an antiseptic mouth wash before expectorating. Mix equal parts of sputum and 3 per cent. sodium hydroxide, shaking well. Incubate the mixture at 38° C. for from fifteen to thirty minutes, until it has become of thin consistency. Then add hydrochloric acid until the mixture is neutral to litmus and centrifugalize at high speed for ten minutes. Pour off the supernatant fluid and inoculate the sediment into tubes containing Petroff's medium. The tubes should be left in the incubator for a few days until the moisture has evaporated or has been absorbed by the medium. Then the cotton stoppers should be paraffined, or they should be removed and sterile paraffined corks inserted in their places. The temperature should be kept at 38.5° C. as constantly as is possible.

Petroff's medium is prepared by mixing one part of meat juice, two parts of egg (both white and yolk), and gentian violet in a quantity sufficient to make 1 part of 10,000 parts of media. The ingredients are prepared as follows:

I. *Meat Juice.*—Infuse 500 gm. of ground beef or veal in 500 cc. of a 15 per cent. aqueous dilution of glycerine. At the end of twenty-four hours squeeze out the meat in a sterile press, collecting the infusion in a sterile glass beaker.

II. *Eggs.*—Allow the eggs to remain in 70 per cent. alcohol about fifteen minutes to sterilize the shells. Break the shells and pour into a sterile beaker. Mix well with a sterile glass rod, and filter through sterile gauze.

III. *Gentian Violet.*—Prepared a 1 per cent. alcoholic solution and add to the medium to give a dilution of 1 : 10,000.

When ingredients have been mixed in the proper proportions, the medium is tubed and inspissated, at 85° C. on the first day in order to solidify it, and at 75° C. on the two following days, for periods of one hour.

The length of time required for a visible growth to appear varies from five to fifteen days, the average being nine to ten days. The growth is seen as a granular, heaped-up, yellowish streak. A slide preparation should be made and stained by the customary technic as outlined above. Should subcultures be desired, it is unwise to make them on medium containing gentian violet, since the dye ultimately retards the growth.

Staining the Influenza Bacillus.—The influenza bacillus stains very slowly with ordinary dyes. It is small and irregular in size, especially in length. It may appear as a very small diplococcus or short diplobacillus (Fig. 111). The longer forms stained with Gram's stain may resemble "safety-pins," the ends being a faint violet, the central portion taking the counter stain. The organisms occur most frequently in clumps.

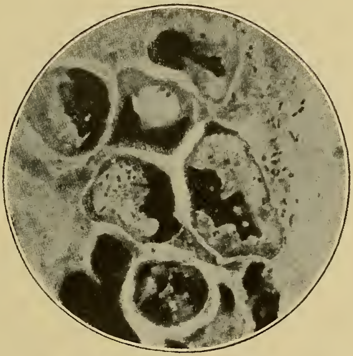


FIG. 111.—Influenza bacilli in sputum. × 1000. (Lord.)

Slides should be made by the "impression method." Gram's stain is recommended. These may be counterstained for five minutes with diluted carbol-fuchsin (*i. e.*, 1 part of carbol-fuchsin plus 9 parts of water or safranin).

Cultivation of Bacillus Influenzæ.—A sterile container should be given to the patient for the collection of the sputum. Two small beakers should be partly filled with sterile normal sodium chloride solution. A dense clump of sputum should be secured with a sterile platinum wire and washed by moving it vigorously about in succession in each dish of sterile salt solution. When the washing has been completed, the mass of sputum is drawn in parallel streaks across the surface of fresh blood-agar plates or tubes, preferably the former. These plates or tubes should be prepared by adding, under aseptic precautions, a few drops of fresh blood to melted agar at 40° C., after which the agar should be allowed to solidify.

The influenza colonies, which are small and have a "dew-drop" appearance, appear in about forty-eight hours. Sub-plants may be made from them to fresh blood-agar tubes. If a growth appears, slides should be examined to ascertain whether or not the organisms have the characteristic morphology and staining appearance.

Ecker's Method.—The bronchial secretions are mixed with 0.5 per cent. solution of sodium taurocholate. After standing for at least twenty minutes, streaks are made on blood-agar plates. Minute, colorless, dew-drop colonies appear after twenty hours' incubation at 37° C. Bile salts have no effect on influenza bacilli, while they dissolve pneumococci and inhibit the growth of other accessory organisms.

Avery's Method.—While the influenza bacillus may be recovered by this method, overgrowth with other organisms is more than likely so that isolation is often difficult. The following method is to be preferred. Avery has called attention to the fact that isolation of the influenza bacillus from nasal or throat cultures is unsatisfactory, since the organism is usually found in association with other bacteria which grow much more luxuriantly. He suggests the addition to the medium of sodium oleate since this prevents the growth of certain Gram-positive organisms, notably the pneumococcus and streptococcus, while the growth of the influenza bacillus is enhanced.

The medium may be prepared as follows: *Two per cent. meat infusion agar* is made up in the usual way. The reaction should be from 0.3 to 0.5 acid to phenolphthalein. The initial hydrogen-ion concentration of the agar should be from 7.3 to 7.5 pH. *A two per cent. solution of neutral sodium oleate* is made in distilled water, sterilized in the autoclave, and kept on hand as a stock solution. Of this solution 5 cc. are added to 95 cc. of the agar, giving a concentration of .1 : 1000. *Sterile human blood or rabbit's blood* is used. The blood is defibrinated and centrifugalized. The serum is withdrawn with a sterile pipette and the cell residue is made up to the original volume with nutrient broth. The blood suspension should be added while the agar is at about 40° C. The *final formula* calls for the following:

2 per cent. meat infusion agar	94 cc.
2 per cent. solution of sodium oleate	5 "
Suspension of red blood cells	1 "

If nutrient broth be desired, oleate hemoglobin bouillon may be prepared by the same formula by substituting broth for agar.

Cultures should be taken from the nasopharynx by the West tube or from the throat by direct swabbing. They should be streaked on the surface of the medium according to the method outlined elsewhere. (See page 411.) Sputum, obtained in a sterile bottle as free from mouth contamination as possible, may be streaked across a plate with a sterile cotton swab.

Diplococcus Pneumoniæ.—The pneumococcus may be identified in a preliminary way by morphology and staining characteristics. Cover-slips are prepared from the sputum. One should be stained with Gram's stain and one with Hiss' or Welch's capsule stain. The serological type may be determined as outlined in subsequent paragraphs.

The organism is a fairly large, lance-shaped coccus, occurring in pairs (Fig. 112). It is non-motile and possesses no flagellæ. The shape may vary from an elongated, almost bacillary form, to a distinctly spherical one. Short or long chains may be seen. The organisms are Gram-positive and are surrounded by a capsule which usually includes a pair of cocci. The capsule may be absent, however, its presence depending somewhat upon the environment of the organism. The most suitable conditions for capsule development are found when growth has taken place in the body fluids of man or animal. Capsules are usually demonstrable around the organisms found in the sputum, and are not found when growth has taken place in artificial media, except milk or those media containing serum. Even under unfavorable conditions of cultivation, however, capsules may be demonstrated by using serum (such as beef or rabbit-serum) as a diluent when spreading organisms upon the cover-slip preparatory to staining.

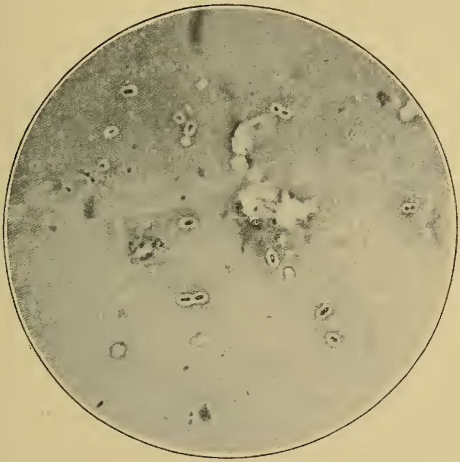


FIG. 112.—Pneumococci in fresh sputum. (Lord.)

The pneumococcus does not grow readily upon ordinary media. The media should be neutral or slightly acid in reaction. Meat-extract media are not as well suited as are media made from a basis of an infusion of fresh meat. Growth may be aided by the addition to the medium of defibrinated blood, or animal or human serum, or by the addition of certain substances such as dextrose and glycerine.

To differentiate the pneumococcus from the streptococcus, the presence or absence of capsules may be helpful. This criterion is not final since pneumococci may show no capsules under unfavorable conditions. As a means of differentiation, two reactions may be used. When Hiss' inulin-serum-water medium is used as a medium, pneumococci break down the inulin causing acid formation and the reddening of the litmus, while streptococci do not. Sterile bile dissolves most pneumococci but not streptococci.

The diplococcus of pneumonia is found occasionally in the mouths of healthy people, in the sputum of acute and chronic bronchitis, at times in the sputum from cases of pulmonary tuberculosis, and in the sputum of pneumonic conditions, where it is a causative factor.

Determination of Type of Pneumococcus.—The determination of type is of great importance since treatment with serum is advisable at present only in infections due to a Type I organism. The facilities needed are simple, including those necessary for bacterial cultivation (media, incubator, etc.), for collection and microscopic examination of sputum, and for mouse inoculation and autopsy. The technic of typing has been developed and described very fully by Avery, Chickering, Cole, and Dochez. Their method is presented here. For typing, the organisms may be recovered from the sputum, spinal fluid, empyema fluid, and by blood culture or lung puncture.

Culture Media for the Pneumococcus.—For the cultivation of the pneumococcus, several requirements should be met: (1) The media should be prepared from infusions of fresh meat and not from beef extracts. (2) The reaction should be from 0.3 to 0.5 acid to phenolphthalein. (3) Excessive heat must be avoided in sterilizing the media. It is preferable to sterilize by the Arnold method on three successive days for twenty minutes each time.

The following directions are given by Avery, Chickering, Cole, and Dochez for the preparation of media:

"One pound of lean chopped beef is allowed to infuse in a liter of tap water over night on ice. The unfiltered infusion is boiled for thirty minutes, filtered through paper, and the loss by evaporation is made up by the addition of water. One per cent. peptone and 0.5 per cent. sodium chloride are now added. 1.5 per cent. agar may be added at this point if agar is desired. The mixture is allowed to boil for two minutes in the case of broth, or, if for agar, until the agar is dissolved. Titration to the neutral point with normal sodium hydrate is then carefully carried out. Media are boiled for six or seven minutes, made up to volume, and filtered clear, and sterilized in the Arnold sterilizer for twenty minutes on three successive days. The media should finally titer 0.3 to 0.5 to phenolphthalein." Bob-veal may be used. Medium may be filtered through glass wool instead of filter paper.

Blood Agar.—Media may be enriched by the addition of small amounts of defibrinated blood, obtained from a rabbit by puncture of an ear vein or by direct heart puncture. The blood should be drawn directly into a sterile container containing small glass beads and defibrinated by shaking. The blood may be kept for a week or more in a refrigerator. Aseptic technic should be employed. Three or four drops of this defibrinated blood are added to 4 or 5 cc. of bouillon or agar.

Sterilization of Bile for Testing the Solubility of Pneumococci.—Fresh ox-bile is obtained from the slaughter house and autoclaved for twenty minutes at 15 pounds pressure. The precipitate which occasionally is seen after heating may be removed by filtering. The filtered bile should be autoclaved again. When bile is not available, a sterile 10 per cent. solution of sodium taurocholate may be used. Solubility is determined by adding to a plain broth culture one-fifth its volume of ox-bile or sodium taurocholate solution.

Procedure for Typing.—Collection of Specimen.—Sputum from the deeper air passages should be expectorated into a sterile, wide-mouthed container, as free from saliva as possible. It should be sent to the laboratory and examined with as little delay as possible. When delay is unavoidable, the sputum should be kept on ice.

Preliminary Examination.—Make films and stain them with Gram's stain, with Hiss' capsule stain, and for tubercle bacilli. Avery and his co-workers direct attention to the fact that it is often possible to identify Type III organisms by staining methods, since they possess especially large, distinct capsules.

Mouse Inoculation.—Select a portion of the sputum about the size of a kidney bean and wash it through three or four changes of sterile physiological salt solution in sterile Petri dishes. If the specimen be exceedingly friable or if it be almost free from contaminating organisms, washing may be omitted. The particle of sputum, washed or unwashed as the case may be, is placed in a small sterile mortar and ground up with 1 cc. of the sterile physiological salt solution to form a homogeneous emulsion which will pass through a hypodermic needle.

One-half to 1 cc. of the emulsion is injected by means of a sterile syringe into the peritoneum of a white mouse (Fig. 113). The pneumococcus grows rapidly, while practically all other organisms (with the exception of Friedländer's bacillus, the influenza bacillus, and occasionally others) die. The blood stream is usually invaded early by pneumococci and not by the other organisms. The time required for a sufficient growth in the peritoneum varies from five to twenty-four hours, averaging six to eight hours with Types I, II and III. When the mouse appears ill, a little of the peritoneal exudate is secured by puncturing the peritoneum with a sterile capillary pipette, after sterilizing the skin and making a small incision through it with a sterile knife. The exudate is removed with the pipette and spread upon a slide. It should be stained with Gram's stain and examined to determine if there be a sufficient growth of pneumococci. If the growth be only moderate or if there be many other organisms present, time should be allowed for more abundant growth.

Autopsy of the Mouse.—This should be performed as soon as the mouse dies or is killed. The peritoneal cavity is opened with suitable aseptic precaution and cultures are made from the peritoneal exudate

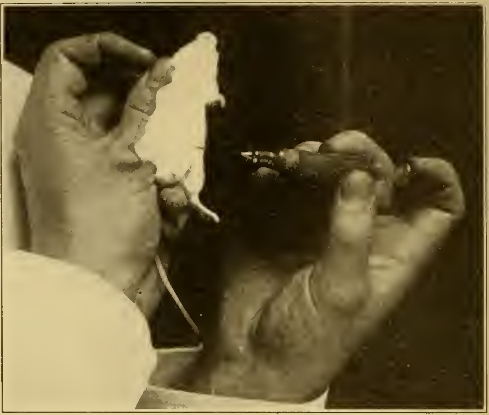


FIG. 113.—Intraperitoneal inoculation of mouse. Rockefeller Institute Monograph No. 7.

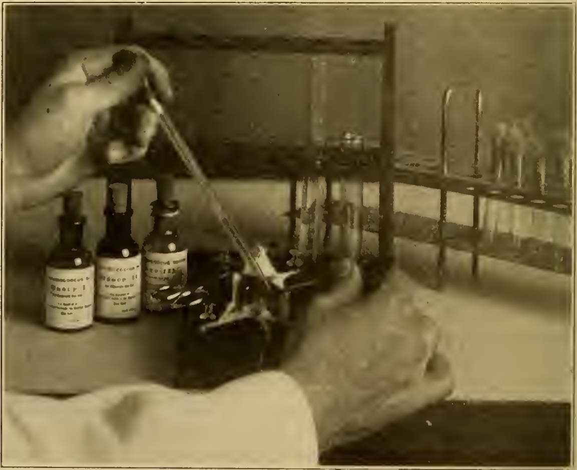


FIG. 114.—Method of collecting peritoneal washings from sputum-inoculated mouse. Rockefeller Institute Monograph No. 7.

in plain or 1 per cent. dextrose broth and on one-half of a blood agar plate. Films are made from the exudate and are stained as with Gram's stain and Hiss' capsule stain. Then 4 or 5 cc. of sterile physiological saline solution are placed in a test-tube. By means of a sterile 1 cc. pipette fitted by a rubber nipple, a small portion of the solution is used for washing out the peritoneum (Fig. 114). The purulent material so obtained is mixed with the bulk of salt solution in the test-tube and the process repeated several times, when the washings are transferred to a sterile centrifuge tube. Finally the heart is opened and cultures are made in plain broth and on the other half of the blood agar plates.

Rapid Cultural Method.—On account of the difficulty in securing white mice, Avery devised a rapid cultural method. A selected purulent kernel of sputum should be washed and emulsified with broth as described in a foregoing section. The emulsion is inoculated into a centrifuge tube containing about 4 cc. of glucose-blood broth.[1]

After inoculation of the glucose-blood-broth, the tube is placed in a water-bath at 37° C. for five hours when a loopful of the culture is streaked on a blood agar plate in order to isolate the pneumococcus in pure culture for subsequent confirmatory determination of type by the agglutination method. The red blood cells are removed by centrifugalizing at low speed for three or five minutes. Erythrocytes should settle to the bottom of the tube and bacteria remain in the supernatant fluid. Three or four cc. of supernatant bacterial suspension are transferred with a sterile pipette to a second sterile centrifuge tube containing about 1 cc. of sterile ox-bile. This is allowed to stand in a water-bath at 37° C. for about twenty minutes, or until the pneumococci have been dissolved. This bile solution of pneumococci is used in the precipitin tests. When bile is not available, the pneumococcus suspension may be used for macroscopic agglutination tests.

Avery found that the results obtained with his method checked very closely with those obtained with the mouse inoculation method, especially when due pains were taken to select suitable portions of sputum, to wash them thoroughly, and to free the pathogenic organisms in the center of the sputum kernels by thorough grinding in the mortar. He thinks, however, that while the results are obtained rapidly, they should be checked by the mouse-inoculation method when mice are available.

Determination of Type of Isolated Organism by the Agglutination Method.—If the organisms be obtained in pure culture, the agglutination method should be used. The tube containing the peritoneal washing is centrifugalized at low speed for a few minutes to throw down cells and fibrin. The supernatant bacterial suspension is pipetted off into another sterile centrifuge tube, which is centrifugalized at high speed to throw down all the organisms. The supernatant fluid, which should be almost clear, is pipetted off and discarded. To the sediment is added sterile physiological saline solution in a sufficient quantity to make a moderately heavy suspension which will have the density of a good eighteen-hour broth culture of pneumococcus. The suspension is used for the agglutination tests, placing 0.5 cc. in each of the small test-tubes. To avoid the confusion of cross-agglutination, proper dilution of sera is important. This varies somewhat with different lots of serum. Five test-tubes are set up as follows:

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[1] Broth is made as outlined in the foregoing paragraphs and to each 100 cc. are added 5 cc. of sterile defibrinated rabbit's blood and 5 cc. of a sterile 1 per cent. glucose solution. A glucose solution should have been sterilized in the Arnold sterilizer by the fractional method at 100° C. on three successive days.

Tube 1	0.5 cc.	Serum I (1: 20 dilution)	+ 0.5	cc.	bacterial	suspension.
Tube 2	0.5 cc.	Serum II (undiluted)	+ 0.5	“	“	“
Tube 3	0.5 cc.	Serum II (1: 20 dilution)	+ 0.5	“	“	“
Tube 4	0.5 cc.	Serum III (1: 5 dilution)	+ 0.5	“	“	“
Tube 5	0.1 cc.	Sterile ox-bile	+ 0.4	“	“	“

The tubes are incubated in a water-bath for one hour at 37° C. When agglutination occurs, there is a rapid clumping of the organisms, which may be observed when the tubes are shaken gently. If there be no agglutination in any of the tubes and if the organisms in tube 5 dissolve with bile, the pneumococcus is classified as a Type IV.

Determination of Type by the Precipitin Method.—When pneumococci are present in the washings from the peritoneum together with a large number of contaminating organisms, agglutination tests are unsatisfactory and the precipitin method should be given preference. The soluble substance liberated by the pneumococci during its growth gives a specific precipitin reaction with homologous anti-pneumococcus serum. The peritoneal washings obtained as described in the paragraph on the "Autopsy of the Mouse," are placed in a centrifuge tube and the tube is run in the centrifuge at high speed to throw down the cells, bacteria, and fibrin. The clear supernatant fluid is pipetted off and used for the precipitin tests. One-half cc. is placed in each of the test-tubes which are set up as follows:

Tube 1	0.5 cc.	Serum I (1: 10 dilution)	+ 0.5	cc.	supernatant	peritoneal	washings.
Tube 2	0.5 cc.	Serum II (undiluted)	+ 0.5	“	“	“	“
Tube 3	0.5 cc.	Serum II (1: 10 dilution)	+ 0.5	“	“	“	“
Tube 4	0.5 cc.	Serum III (1: 5 dilution)	+ 0.5	“	“	“	“

Incubation is usually unnecessary since the specific precipitin reaction occurs at once in the tube containing homologous serum. The contact ring should be noted. The reason for employing two dilutions of Serum of Type II is to distinguish between the Type II pneumococcus and its sub-groups. The sub-group pneumococci give a reaction only with the undiluted serum while the pneumococci of Type II give a precipitation in both tubes. When a negative reaction occurs in all tubes, the organism is classed in Group IV.

Confirmation of Findings.—The results obtained with the peritoneal washings should be regarded as only preliminary and should be confirmed by carrying out similar tests with the pure culture obtained from the heart's blood. The technic is the same as that outlined for peritoneal washings.

Determination of Type III Pneumococcus.—Pneumococcus mucosus (or Type III) can often be identified on morphological and cultural grounds. It is larger, not so definitely lanceolate, and broader than the other types (Fig. 115). The capsule is large and stains well with the capsule stains, often showing with the counterstain of Gram's stain. Cultures on blood-agar are moist, mucoid, and confluent, and the peritoneal exudate of the infected mouse is also sticky and mucoid.

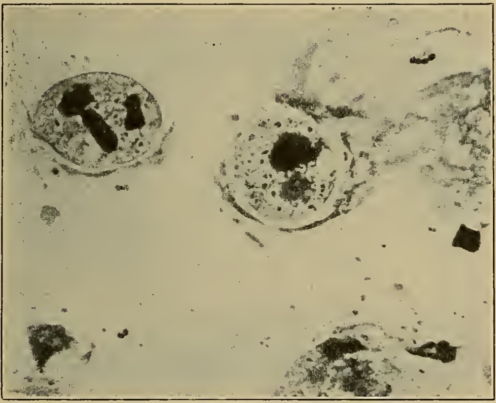

FIG. 115.—Streptococcus mucosus capsulatus in sputum. (Lord.)

Micrococcus Catarrhalis.—This organism is found occasionally in the sputum of patients suffering with catarrhal affections of the upper respiratory tract. It is only slightly pathogenic. On morphological grounds it cannot be distinguished from the gonococcus or meningococcus, for it is a small, biscuit-shaped, Gram-negative diplococcus (Fig. 116). Its ready growth upon ordinary media, however, furnishes a point of differentiation from the gonococcus, which grows only upon special media. Differentiation from the meningococcus is not so easy, since this coccus occasionally grows readily upon ordinary media. The growth of the micrococcus catarrhalis upon ordinary culture media is heavier and the colonies are granular and distinctly white, while those of the meningococcus are translucent, finely granular, and grayish-white. Agglutination reactions are helpful in making a differentiation. The differentiation assumes great significance in the examination of cultures from the nose and throat.

Streptococcus and Staphylococcus.—Streptococci and staphylococci of all varieties may be found in the sputum, not only in cases of acute and chronic bronchitis, but as secondary invaders in advanced tuberculosis. Streptococci may be found in acute lobular and bronchopneumonia, where they may be the causative factor. When one desires to cultivate the streptococci and to study the cultural characteristics,

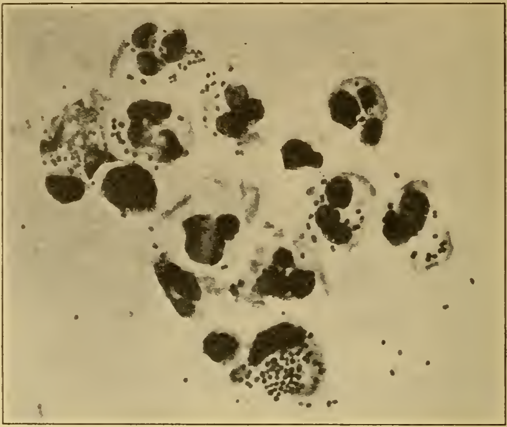

FIG. 116.—Micrococcus catarrhalis in smear from sputum. (Lord.)

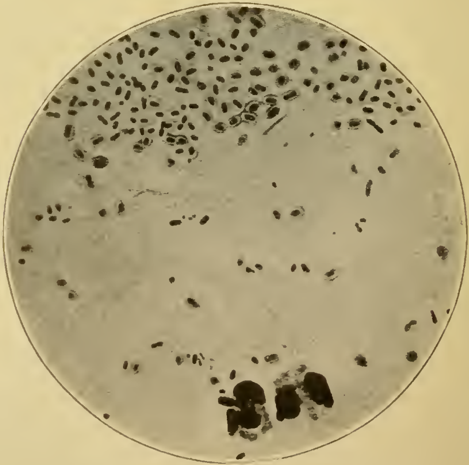

FIG. 117.—Friedländer's bacillus in sputum. (Lord.)

the sputum should be washed and ground to an emulsion as directed in the foregoing paragraphs which describes the typing of pneumococci. Cultures may then be made in ordinary media and on blood agar. (See Bacteriology of Blood.)

Bacillus Mucosus Capsulatus.—Friedländer's bacillus is present in a comparatively small number of the cases of lobar pneumonia. The infection is extremely severe and usually fatal. The organism is a short, plump bacillus, some of the shorter and thicker forms resembling cocci (Fig. 117). It is non-motile, forms no spores, stains readily with ordinary stains, and Gram-negative. The thick capsule is clearly marked and is readily demonstrated when the organism has been obtained from body exudates, often when grown upon agar. The bacillus is readily cultivated upon ordinary media.

Bacillus Smegmæ.—The smegma bacillus is of importance from the standpoint of differentiating it from the tubercle bacillus. Its occurrence has been reported in the sputum and in the material squeezed out of the tonsillar crypts. While acid-fast, the bacilli do not retain the stain in the presence of acid as tenaciously as tubercle bacilli. It is generally thought that Pappenheim's rosolic acid method of decolorizing is adequate for differentiation.

THE SPUTUM IN DISEASE.

Acute Bronchitis.—In the earlier stage of acute bronchitis, the sputum is white, contains a few leukocytes and red blood cells, epithelial cells, and mucus, and often a number of varieties of organisms. Later, the color changes to a yellow or even a greenish tint and microscopic examination shows many leukocytes and a few red blood cells. Smears made directly from the sputum may show many types of organisms, but often only one or two types of microörganisms are found after the purulent mass is washed and ground to obtain only the organisms from the lower portions of the respiratory tract. The possible offending organisms include the pneumococcus, streptococcus, micrococcus catarrhalis, the influenza bacillus, and occasionally Friedländer's bacillus or even the colon bacillus. In making a diagnosis of influenza, it is advisable to culture the organism from the sputum.[1]

Chronic Bronchitis.—The sputum is extremely variable in both amount and appearance. In some cases, there is none. Frequently it is abundant, mucopurulent or purulent in appearance, yellow or green in color, and contains many degenerated leukocytes and alveolar epithelial cells. Practically any of the organisms mentioned as possible invaders in bronchitis may be found.

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[1] The role which the influenza bacillus played in the great epidemic of the latter part of 1918 is a moot question. Lord, Scott and Nye found 78 per cent. positive influenza cultures in epidemic cases. Pritchett and Stillman using Avery's oleate blood agar medium, cultivated the B. influenzæ in 93 per cent. of the cases of influenza and bronchopneumonia. In interpreting these results, it is to be borne in mind that the influenza bacillus is a common invader of the respiratory tract, that it is found in normal individuals, and that it often occurs even in cases of pulmonary tuberculosis with the tubercle bacillus.

Bronchial Asthma.—The sputum is usually thick and tenacious and often is expectorated in the form of little balls or gelatinous masses, the "perles" of Laennec. Curschmann's spirals, eosinophilic cells, and Charcot-Leyden crystals are often seen.

Acute Lobar Pneumonia.—In the early stages, the sputum may be mucoid in character but the appearance soon changes and it becomes blood-tinged, "rusty," tenacious or sticky. It is frequently very viscid. Occasionally the sputum may be fluid and brown in color, when it is spoken of as "prune-juice sputum." During the stage of resolution it becomes yellow, thinner and more abundant, and the cells are more numerous. At times it is green. The pneumococcus may be seen in cover-slip preparations when suitably stained and may be cultivated by methods which have been described.

Gangrene of the Lung.—The sputum has a very fetid, offensive characteristic odor. It is usually fluid and separates into three layers, of which the lower is a heavy sediment; the middle, fluid and green or brown in tint; and the upper, frothy and thick. At times portions of lung tissues are expectorated. Microscopically, fatty acid crystals, bacteria, a few cells, granular material, and elastic tissue are found.

Fibrinous Bronchitis.—In chronic idiopathic bronchitis, casts of a certain portion of the bronchial tree may be expectorated. At times the casts are rolled up and mixed with blood and mucus, or they may be seen as large branching structures. They consist mainly of fibrin, and contain leukocytes and epithelial cells. Fibrinous bronchitis is seen occasionally in the course of some of the acute infections, notably diphtheria, typhoid fever, pneumonia and the eruptive fevers.

Chronic Passive Congestion.—The sputum is often tenacious and blood-tinged. On microscopic examination, "heart failure cells" and red blood cells may be demonstrated.

Acute Pulmonary Edema.—The sputum is copious, frothy, and is often tinged a bright pinkish color from the admixture of blood.

Malignant Disease of the Lung.—There is nothing especially characteristic about the sputum though occasionally bits of the new growth are expectorated. When found, they should be sectioned and examined histologically.

Pulmonary Tuberculosis.—The sputum varies greatly in quantity, appearance, and in the number of tubercle bacilli which it contains. In acute miliary tuberculosis it may be slight in amount, similar in appearance to that seen in acute bronchitis, and devoid of tubercle bacilli. In early cases of pulmonary tuberculosis, the quantity is often small, and expectoration may be limited to the early morning. Blood streaks are seen at times; indeed, a profuse hemorrhage may be the first symptom. As the lung tissue breaks down sputum is mucopurulent, more copious in amount and contains elastic tissue and broken down lung tissue. Advanced cases in which cavity formation has taken place show so-called "nummular sputum," spherical masses of pus floating in thin fluid. Such sputum often has an offensive odor, and the purulent portions may contain large numbers of tubercle bacilli.

CHAPTER VI.

EXAMINATION OF BODY FLUIDS, EXUDATES AND MISCELLANEOUS METHODS.

EXAMINATION OF SPINAL FLUID.

Technic of Spinal Puncture.—The puncture must be carried out under aseptic conditions. Especial attention should be given to the preliminary arrangement of the patient, who lies on his right side on the extreme edge of the bed, with the lower portion of the back as nearly straight in both planes as possible, and the knees drawn up so that the thighs are at right angles to the trunk. Both shoulders should be at right angles to the surface of the bed, in order to prevent a sagging of the body and consequent curving of the spine in the lumbar region.

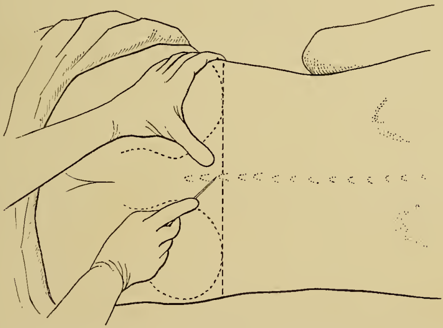

Fig. 118.—Showing point for lumbar puncture in median line in the first interspace below the level of the iliac crests. It is impossible to show the correct position of the needle, which should be inserted at a right angle in both planes.

Often a small pillow placed in the curve between the crest of the ilium and the costal margin is useful in keeping the spine straight. The skin of the entire lumbar area is carefully cleansed by scrubbing with tincture of green soap, ether, alcohol, and finally by applying tincture of iodine. Anesthesia is produced by infiltrating the skin and subcutaneous tissues with $\frac{1}{100}$ solution of procain by means of a sterile all-glass syringe. The procain solution may be sterilized by boiling. For the puncture, a needle fitted with an obturator is desirable.

The intervertebral space at the level of the iliac is the one usually selected for puncture, though sometimes the space immediately above is somewhat easier to enter (Fig. 118). The lumbar puncture needle is inserted in the *midline* toward the lower border of the intervertebral space, keeping it at *right angles to the body in both planes,* when it should pass into the canal without touching bone in its course. If the space below the line of the iliac crests be selected, the point of the needle should be directed slightly toward the head. When it is felt that this space has been entered, the obturator should be withdrawn. Should no fluid appear, the obturator is replaced and the needle inserted a little farther. Failure to obtain fluid is often due to the fact that the point of the needle has been directed downward or to the right of the canal so that the needle may be partially withdrawn and the butt end depressed in order to raise the point of the needle, which on reinsertion usually reaches the desired destination. Unless pressure be greatly increased or serum be injected, not more than 7 or 8 cc. should be removed. (For an excellent discussion of methods of performing lumbar puncture, see article by Joseph C. Regan.)

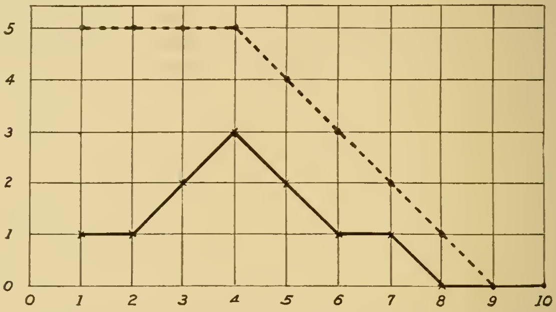

Fig. 119.—Colloidal gold reaction curves. Broken line represents paretic curve; solid line, curve in luetic zone. Tube numbers are represented on abscissa line and depth of color change on ordinate line.

Precautions.—When brain tumor is suspected, much care should be exercised not to decompress too rapidly and to take only a small amount of fluid. Under all circumstances, the patient should be kept flat on the back or side in bed for at least twenty-four hours, not raising the head from the pillow for any purpose.

With all possible precautions, a severe headache, the so-called "puncture headache," appears occasionally. This may last for days, even as long as three weeks. Apparently there is no effectual method of treatment. The patient is usually perfectly comfortable when in the recumbent posture but as soon as the head is raised complains of a throbbing sensation at the base of the skull and back of the neck and at times of nausea and vomiting.[1]

If a *determination of pressure* be desired, a long piece of glass tubing with internal diameter of 1 mm. may be attached to the needle by a piece of rubber tubing. The spinal fluid is allowed to ascend as far as it will go. The height to which it ascends may be marked on the tube by an assistant with adhesive tape or a wax pencil and the distance measured in millimeters. The determination of pressure is hardly worth while from a practical point of view. When it is greatly increased, as in tuberculous meningitis, the fact is quite apparent, since the fluid spurts out in a stream, often for a distance of several feet. Normally it comes out drop by drop, though the first cubic centimeter or so may come in an almost continuous stream. The manometer readings are not absolutely accurate since the level of the fluid depends greatly upon the relative position of the patient's head.

Routine Examination of the Spinal Fluid.—The procedure varies somewhat with the clinical findings. When syphilis of the central nervous system is suspected, the following would be an appropriate order for the tests:

1. Gross appearance (color, turbidity, presence of blood, etc.).
2. Cytological examination.
3. Chemical examination (globulin and Lange tests).
4. Wassermann reaction.

When an acute infective meningitis is suspected, the following should be the order of the examination:

1. Observation of gross appearance (color, turbidity, presence of macroscopic pus, etc.).
2. Inoculation of media.
3. Removal with a sterile pipette of about 1.5 cc. for cell count.
4. Centrifugalization of a portion for a cytological and bacteriological study of cells.
5. Incubation of remainder of uncontaminated fluid in original container at 37° C.

GROSS APPEARANCE.—*Color.*—Normal spinal fluid is colorless, and in the gross cannot be distinguished from distilled water. Its specific gravity is given as 1.006 to 1.007. *Xanthocromia* (canary-yellow tint) may be seen after intracranial hemorrhage. It is usually due to changed blood, and is generally accompanied by an increased amount of globulin. Nonne's syndrome (yellow color, no increase in the cell count, and an excess of protein), may be produced by localized obliteration of the pia-arachnoid space, forming a lower cul-de-sac with no communication with the space above.

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[1] A plausible theory has been advanced by MacRobert to account for these headaches. The dura is a rigid, tough membrane without elastic tissue. When it has been punctured, the hole persists, sharp and clean-edged, unless closed by soft arachnoid tissue which may fill it and so block it. This closure may not occur since the subarachnoid tissue may be dragged through the hole by the departing needle and so form a spout or wick for the drainage of the cerebrospinal fluid. This constant drainage permits a marked diminution in the amount of fluid. and the base of the brain is deprived of its supporting cushion of fluid.

Turbidity.—When turbidity is present, it is due to a high cellular or bacterial content. In most cases of cerebrospinal syphilis, the fluid is clear, but distinct turbidity may be seen with the high cellular content which sometimes accompanies active syphilitic meningitis. Fluids of this type may present the opacity of ground glass.

Blood.—Blood may be seen when the tissues have been injured by the needle; in cases of cerebral hemorrhage when the area of hemorrhage has communicated with the subarachnoid space; and after fracture of the skull. Fluid drawn a considerable time after hemorrhage may show a canary-yellow color.

Cytology.—The cells may be enumerated by the following method (Swift and Ellis): The hemocytometer commonly used for enumerating white blood cells and the ordinary hemocytometer chamber are employed. Staining fluid (methyl violet, 5 B., 0.1 gm.; glacial acetic acid, 10 cc.; distilled water, q. s. to 100 cc.) is drawn to 1.0 mark and then aspirated into the pipette to coat the walls of the bulb. The pipette is filled with freshly drawn spinal fluid to the 11 mark, the mixture is thoroughly shaken and set aside for about fifteen minutes. At the end of this time the lymphocytes will have been stained sufficiently. Ten fields, each 10 mm. square and 0.1 mm. deep, are counted, following the routine described by the enumeration of leukocytes (see page 38). The total, multiplied by $\frac{10}{9}$ to correct for the dilution, gives the number of cells per cm.

The count will be reduced considerably unless it be made very soon after the withdrawal of the fluid, since the lymphocytes disintegrate readily. The normal count varies from 1 to 6. An increased count is referred to as *pleocytosis*.

Differential Count.—The fluid is centrifugalized for fifteen or twenty minutes when the supernatant fluid is withdrawn with a capillary pipette. The residual drop is placed upon a slide, dried, fixed and stained with Wright's stain. The essential points in the technic are: slow drying of the sediment in the air with little or no heat; fixation with absolute methyl alcohol for two minutes. The application of the flame should be avoided to prevent rapid drying of the cells of lymphocytic type. Normally, only lymphocytes are seen.

Chemical Examination.—*Reduction of Fehling's Solution.*—Normal spinal fluid contains a substance, presumably a sugar, which reduces Fehling's solution. It is absent in meningitis, but may be present in slight inflammatory conditions. Absence of reduction is of uncertain import. The test has little practical value.

Globulin.—Globulin is found in normal spinal fluid, but only in traces, increased amounts accompanying practically all inflammatory conditions.

Noguchi Butyric Acid Test.—Mix 0.2 cc. of spinal fluid (free of blood) in a test-tube with 1.0 cc. of 10 per cent. dilution of butyric acid in normal saline solution. Heat this to boiling and add 0.2 cc. of normal sodium hydrate. Heat the mixture again to boiling, and cool. Normally a slight opalescence is obtained. A distinctly granular or flocculent sediment indicates an increase in globulin. The importance of using absolutely pure butyric acid has been emphasized by Ross and Jones, who secured a large number of faulty negative results with the Noguchi test because of the use of impure acid.

An arbitrary standard for reading the Noguchi reaction has been suggested by Swift and Ellis:

Negative: opalescent to very faint haze.
Plus-minus: faint haze to haze.
One plus: fine granular precipitate.
Two plus: heavy granular or coarse flocculent precipitate.
Three plus: very heavy flocculent precipitate.

Pandy's Test.—To about 1 cc. of a saturated aqueous solution of phenol is added one drop of spinal fluid. In the presence of an increased quantity of globulin, a bluish-white ring or cloud is formed immediately.

Ross-Jones Modification of Nonne's Test.—In a test-tube are placed about 2 cc. of a saturated solution of ammonium sulphate. One cc. of spinal fluid is run into the test-tube from a pipette to form a layer over the ammonium sulphate solution. A white or gray ring marking the point of contact constitutes a positive reaction.

Lange Colloidal-gold Reaction.—Normal spinal fluids will cause no change in properly prepared solutions of colloidal gold when suitably diluted with 0.4 per cent. solution of sodium chloride, which is of the proper concentration to keep the protein in solution. Abnormal fluids bring about partial or complete precipitation of the colloidal gold, producing color changes which may be graphically charted and are apparently specific for certain conditions.

The following technic has been adapted from that published by Miller, Brush, Hammers, and Felton, with only minor changes. After a trial of many other methods, the writer has adopted this for his own use and has found it highly satisfactory.

Solutions Required.—(1) Solution of colloidal gold, which must answer the following requirements:

(*a*) It must be neutral to alizarin on the day it is used.

(*b*) It must be absolutely transparent and of a brilliant orange-red or salmon-red color.

(*c*) It must be "unprotected," that is, 5 cc. should be completely precipitated in one hour by 1.7 cc. of 1 per cent. sodium chloride solution.

(*d*) It should give a typical paretic curve with a known paretic spinal fluid.

(*e*) It should give no more than a No. 1 reaction in any tube with a known normal spinal fluid.

(2) Sodium chloride solution, sterile, 0.4 per cent. prepared from C. P. NaCl and distilled three times.

Preparation of Solutions.—The chemicals must be weighed with extreme accuracy. Lack of attention to detail will bring only failure.

Apparatus Required:

Pyrex beakers, capacity 500 cc.

Pyrex beakers, capacity 1000 cc. for making solution.

Pyrex Florence flasks, capacity 2000 cc. for collecting water.

Graduates, 100 cc. and 500 cc.

Pipettes, two, 1 cc., graduated in $\frac{1}{100}$ cc.

Pipettes, four, 5 cc., graduated in $\frac{1}{20}$ cc.

Pipettes, four, 1 cc., one mark only.

Pipettes, four, 10 cc. graduated in $\frac{1}{10}$ cc.

Thermometer of Jena glass.

Bunsen burner, tripod, wire gauze with asbestos center.

Copper still, tin-lined, with worm condenser of block-tin, including suitable supports and 4-tube Bunsen burner.

Thick walled lipless test-tubes, about $\frac{11}{16}$ inch in diameter by 6 inches long.

Rack with 12 holes of size to receive the test-tubes specified.

Cleaning Glassware.—This is an essential step in the technic. Beakers, flasks, pipettes, test-tubes, and graduates are treated first with a small amount of nitric acid, which is allowed to come in contact with the entire inner surface of the various vessels. This readily removes any precipitate. Tap-water is then used copiously for rinsing, which is continued with 2 or 3 changes of once-distilled water and finally rinsed with a little triple-distilled water just before use. The pipettes are drained by allowing them to stand upright on a piece of clean filter-paper.

Reagents Required.—1. *Triple-distilled Water.*

The water which is used in making the solutions should be distilled three times. The same flask should be used throughout for receiving the distilled water. On each distillation, the first 100 cc. of distillate should be discarded.

2. *Gold Solution.*—Gold chloride crystals should be obtained in small, hermetically-sealed glass ampoules.

Gold chloride (Merck's yellow crystals)	1 gm.
Water, triple-distilled, to	100 cc.

This solution keeps for months when placed in a dark glass-stoppered bottle away from the light.

3. *Potassium Carbonate Solution.*—This should be made up immediately before use.

Potassium carbonate (Merck's desiccated blue label)	2 gm.
Water, triple-distilled, to	100 cc.

4. *Formaldehyde Solution.*—Merck's Blue Label (highest purity) 40 per cent. solution formaldehyde should be used. A neutral reaction may be maintained by placing powdered marble-dust in the bottom of the bottle. The bottle is shaken occasionally and the powder

allowed to settle before use. The folowing solution should be made up just before use:

Formaldehyde (40 per cent. solution)	1 cc.
Water, triple-distilled, to	40 "

5. *Oxalic Acid Solution.*—This solution also must be prepared just before it is needed for use in making up the colloidal-gold solution.

Oxalic acid (Merck's blue label, crystals)	1 gm.
Water, triple-distilled, to	100 cc.

Preparation of the Colloidal-gold Solution.—In a beaker heat 1000 cc. of triple-distilled water, using a Bunsen flame, and protecting the beaker with asbestos-filled iron gauze. When the temperature reaches 60° C., add 10 cc. of the gold chloride solution and 7 cc. of the potassium carbonate solution. At 80° C., stir the solution and add 10 drops of the oxalic acid solution. At 90° C., add the formaldehyde solution slowly, drop by drop, using only enough to produce an initial pink color. Usually about 5 cc. are required. The flame should be reduced to a point sufficient to keep the temperature at 90° C. until the color change appears, when it is removed.

If satisfactory, the solution should correspond to the standards previously described. A yellow shimmer indicates an unsatisfactory solution. The solution should be transparent so that if a beaker containing 7.5 cm. of the solution be placed over a book-page, ordinary type may be read through the solution.

When the correct amount of alkali has been employed, the solution usually remains colorless until it has reached a temperature of 90° C. When the solution, however, becomes definitely pink after the addition of oxalic acid, the formaldehyde should be added slowly and only until the color is intensified. If, on the other hand, no color appears after the temperature has been held at 90° C. for some time, formaldehyde should be added drop by drop until a pink color is evident. When this color fails to appear, potassium carbonate solution may be added drop by drop until it is seen.

Neutralization of the Solution.—When the solution answers all other requirements but is not neutral in reaction, it may be neutralized. The indicator to be employed is a 1 per cent. solution of alizarin red in 50 per cent. alcohol. Added to a neutral colloidal gold solution, this gives a brownish-red tint, to an acid solution, a lemon-yellow color, and to one which is alkaline, a purple-red. Two drops of the indicator are placed in a test-tube with 5 cc. of the colloidal gold solution. If the color be brownish-red, the solution is satisfactory from the standpoint of reaction. If not, titration may be conducted in the following way. A rack is prepared with ten test-tubes, into each of which is placed 1 cc. of distilled water. Into the first test-tube, place 1 cc. $\frac{N}{50}$ hydrochloric acid if the reaction of the solution be alkaline, or 1 cc. of $\frac{N}{50}$ sodium hydrate if it be acid. Draw the contents of the first test-tube into a pipette and eject into the test-tube. Repeat this two or three times to insure thorough mixing and then place 1 cc. in the second tube. Mix the contents of the second tube by drawing the fluid up into the pipette and ejecting it into the test-tube several times, and then place 1 cc. in the third tube. Repeat this procedure with each tube, omitting the tenth tube, and discarding 1 cc. of the mixture in the ninth tube. The amount of acid or alkali in each tube is readily calculated. In the first we have 0.5 cc. $\frac{N}{50}$ solution, in the second 0.25 cc., in the third 0.125 cc. and so on down. The tenth tube, containing only colloidal gold solution, serves for color comparison. To each tube add two drops of the indicator and 5 cc. of the colloidal gold solution to be tested. Select the first tube in which the neutral reaction (brownish-red tint) is obtained. This indicates the amount of acid or alkali which will have to be added to 5 cc. of solution to secure a neutral reaction. Divide this amount by 5 to find the amount necessary to neutralize 1 cc. and multiply it by the amount of solution to be neutralized. The corresponding amount of $\frac{N}{50}$ acid or alkali should be added slowly while agitating thoroughly. Solutions should not be neutralized until they are at least forty-eight hours old.

Performance of the Reaction.—When a satisfactory solution is available, the performance of the reaction itself is comparatively simple. Only uninfected, blood-free spinal fluids should be examined.

Eleven test-tubes should be set up in a rack. In the first tube place 1.8 cc. of 0.4 per cent. NaCl solution and 0.2 cc. of the spinal fluid to be tested. In each of the remaining tubes put 1 cc. of 0.4 per cent. NaCl. Mix the contents of the first tube well and transfer 1 cc. of the mixture to the second tube. Mix this, and with the same pipette take 1 cc. and put in the third tube. Continue this with each tube up to and including the tenth. When the contents of this tube has been mixed, withdraw 1 cc. and discard it. The eleventh tube, containing only 0.4 per cent NaCl solution and no spinal fluid, serves as a control. Then add to each tube 5 cc. of the colloidal gold solution. Shake each tube separately and thoroughly. Set the rack aside, and take readings in thirty minutes and again in twenty-four hours.

Readings.—In making the readings, each tube is given a number on a scale of 5 according to the extent of the color change. The following is the scale:

Colorless	5
Almost colorless, but a tinge of blue	4
Blue	3
Lilac or purple	2
Reddish-blue	1
Original salmon-red (unchanged)	0

The readings are then recorded in the order in which the respective tubes stand: *e. g.*, 5, 5, 5, 5, 4, 3, 2, 1, 0, 0, represents a curve in the "paretic zone," while within the "luetic zone" would be: 1, 1, 2, 3, 2, 1, 1, 0, 0, 0, or 2, 2, 3, 4, 4, 3, 1, 0, in other words, where the greatest color change is reached in third, fourth or fifth tubes (Fig. 119). A curve is referred to as falling within the "meningitic zone" when the depth of the color reaction is in the lower dilutions.

Interpretation of the Findings.—The curve obtained in tabes and cerebrospinal lues fall in approximately the same zone. In treated cases the writer has sometimes found that the curve persisted in the luetic zone after the disappearance of the other laboratory findings. The paretic type of curve has been found in cases of multiple sclerosis where the Wassermann reaction was negative. The reaction is of distinct value in differentiating between paresis and syphilitic meningitis.

Wassermann Reaction with Spinal Fluid.—The Wassermann reaction is of assistance in the diagnosis of syphilitic affections of the central nervous system. Fortunately it is possible to employ much larger quantities than is the case with blood serum because of the fact that the anticomplementary power of the fluid is light. By the use of graduated amounts, the reaction is placed upon a quantitative basis which is helpful in following the progress of treated cases. The technic for the Wassermann reaction with spinal fluid has been given elsewhere (see page 151).

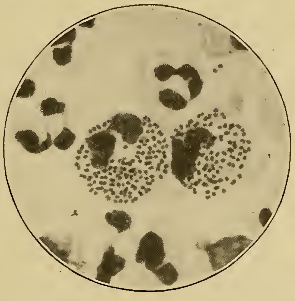


FIG. 120.—Meningococci in pus. × 1000. (Kendall.)

Bacteriological Examination of the Spinal Fluid.—The fluid should have been drawn into a sterile test-tube. When a bacterial infection is suspected, it is wise to remove a small quantity of the fluid with a sterile pipette and place it in a separate tube. This fraction can be used for the enumeration of the cells, for the globulin test, and to make slides for differential count and preliminary search for bacteria. Cultures should be made upon appropriate media with some of the remainder. A considerable portion of the fluid should then be placed in the incubator and kept at 37° C. for twenty-four to forty-eight hours. The fluid itself serves as an excellent culture medium. Cultures may be made from this, since the organisms multiply in the fluid, and cover-slips may be prepared for staining. Organisms are often found in this incubated fluid when none can be found in the fresh fluid.

Infection with Micrococcus Intracellularis Meningitidis.—Usually, the fluid is turbid and even purulent. At times it may be so thick that it will scarcely run through a wide-bore needle. The organism is a biscuit-shaped diplococcus, usually occurring in pairs but occasionally in fours or even in small masses. In form it resembles the gonococcus. It may be found both within and without the cells, and it is thought that the prognosis is better when it is intracellular (Fig. 120). It stains well with ordinary stains and is Gram-negative. Well-defined capsules are not demonstrable. The findings of a Gram-negative diplococcus in turbid spinal fluid containing a preponderance of polynuclear cells generally means infection with the meningococcus, although it is well to confirm this with cultures.

Occasionally clear or almost clear spinal fluids show on microscopic examination polymorphonuclear and mononuclear cells in almost equal numbers and no organisms. Such fluids should be placed in the incubator, since often organisms are found in large number on the following day.

For cultures, a suitable medium is glucose-ascitic agar, made with a meat infusion basis and with reaction neutral to phenolphthalein. In emergencies, Loeffler's blood serum may be used, or a few drops of whole human blood, obtained under sterile conditions, may be added to glucose agar which has been melted and whose temperature has been reduced to 40° C. This medium is poured into a sterile Petri dish and allowed to solidify. Some of the spinal fluid is streaked across the plate with a platinum loop when the fluid is fairly turbid, while a larger quantity should be run over the surfaces of the plate from a sterile pipette when the fluid is clear. The colonies are rather minute, grayish-white in color, and not confluent.

Infection with Diplococcus Pneumoniæ.—This is a more frequent complication of pneumonia than is generally recognized. The spinal fluid is usually turbid, occasionally purulent. The cells are increased in number, polymorphonuclear leukocytes predominating. The morphology and staining reactions of the organism have been described elsewhere (see page 360). In general terms, a Gram-positive, encapsulated lance-shaped diplococcus, in all reasonable probabilities can be only a pneumococcus. Confirmation may be made by cultural methods as described in the chapter on Sputum (see page 362). The fluid may be centrifugalized if desired and the sediment taken up with 1 cc. of sterile normal salt solution after decanting the supernatant fluid. This may be injected into a mouse for the purpose of determining the type of the organism. Dextrose blood broth may be inoculated with spinal fluid, and the cultures used for typing according to Avery's method.

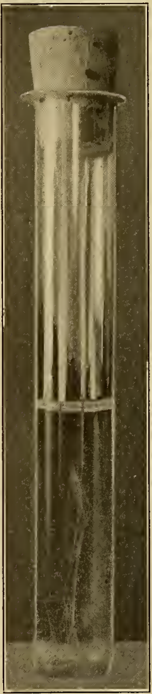
Infection with B. Influenzæ.—The influenza bacillus is occasionally found in the spinal fluid, usually following infection elsewhere. Its morphology and staining have been described (see page 359). Cultures may be made by planting fairly large quantities, 1 or 2 cc., on blood-agar plates.

Infection with B. Tuberculosis.—When tuberculous infection is suspected, it is usually possible to secure a fairly large quantity of fluid. A part should be poured or pipetted off and used for the cell-count, globulin test, and Lange reaction. Another portion should be centrifugalized at a rapid rate. The supernatant fluid should be poured or pipetted off. The sediment is mixed with a drop or two of the fluid in the centrifuge tube, spread upon a cover-slip with the platinum loop, dried carefully, fixed by covering with absolute methyl alcohol for two minutes, and stained by the Ziehl-Neelsen method (see page 436).

The remainder of the fluid is allowed to stand in the incubator at 37° C. for about twenty-four hours. A fibrin clot usually forms and attaches itself to the sides and bottom of the tube, entangling the tubercle bacilli in its meshes (Fig. 121). A clot is withdrawn with a sterile platinum wire, is teased out on a slide, dried, fixed with heat, and stained for tubercle bacilli in the usual manner.

A variation in technic which gives excellent results is to pour the spinal fluid into a short sterile graduate or into a small sterile Stender dish. The vessel must be large enough to permit a cover-slip to rest flat in the bottom. The fluid is allowed to stand in the incubator for about twenty-four hours. The cover-slip is then removed from the bottom of the container by lifting it up with a platinum wire bent to form a wide loop. This loop is slipped under the cover-slip and the cover-slip is raised without tilting it, keeping it in a horizontal plane. The fibrin clot will adhere to its upper surface, and will not require teasing out. After drying and fixing, it may be stained in the usual way.

Infection with Other Organisms.—Other organisms which may cause meningitis are the streptococci, the staphylococci, the colon bacillus, the typhoid bacillus, the Bacillus pyocyaneus, Micrococcus tetragenous, yeasts, etc. In almost all instances, meningitis due to these invasions by organisms is secondary to infection elsewhere, such as otitis media, mastoid infection, infections of the nasal cavities or accessory sinuses, wounds of skull or scalp, endocarditis, erysipelas, etc.



.Fig. 121.—Fibrin clot in spinal fluid in tuberculosis.

Significance of the Findings.—*Cell-count.*—Increased cell-count is found in any active meningitis. In making a differential count of the cells, the polynuclears will be found to predominate in the infections due to the pneumococcus, the meningococcus, and the pyogenic organisms, while in tuberculosis the lymphocytes comprise 90 to 95 per cent. of the cells. This rule, however, is not a rigid one. For example, when the infection with meningococcus has passed into a subacute stage, the polymorphonuclear cells may be equaled or exceeded in number by the lymphocytes. In syphilitic infections, the cells are practically all small round cells of lymphocyte type.

Globulin.—Increased globulin is due to transudation from vessels of the central nervous system. It may be seen in tumors of cord or brain, of bony canal or of meninges, in arteriosclerosis, or any inflammatory condition of the central nervous system.

Wassermann Reactions.—A positive reaction is found only in the spinal fluid when syphilitic involvement of the central nervous system is present.

Lange Colloidal-gold Curve.—The findings have been given in the preceding paragraphs describing the reaction.

Characteristic Findings (Tuberculous Meningitis.)—The fluid is usually absolutely clear and under increased pressure, globulin is increased, and cell-count is high. A differential cell-count usually shows predominance of lymphocytes, though this is not invariable, for polymorphonuclears are occasionally formed in the majority, especially in the early stages. Tubercle bacilli may be found.

Syphilitic Infections (paresis, tabes dorsalis, syphilitic meningo-endarteritis).—The blood Wassermann reaction is often positive. This is not constant and a negative Wassermann reaction in the blood does not exclude syphilis of the nervous system. The spinal fluid is usually clear. It may be under slightly increased pressure. Usually the Wassermann reaction in the spinal fluid is positive, cell-count increased and globulin in excess of normal. The Lange colloidal-gold curve is helpful in differentiating between these conditions.

Acute Anterior Poliomyelitis.—The fluid may be clear, slightly hazy, or even flocculent. The cells are usually increased, the count ranging from 0 to 2000 per c.mm. The globulin may be greatly increased. The differential count usually shows above 50 per cent. polymorphonuclears in the early stage, while later there are usually 90 per cent. or more mononuclears. In general, characteristics of the fluid depend to some extent upon the severity of the process.

Other Infections.—The fluid is usually purulent. With any acute infection, the globulin is increased and the cell count high. The differential count usually shows a vast majority (90 per cent. or over) of polymorphonuclear cells. When the infection becomes chronic, the mononuclears often become more numerous, and may predominate over the polynuclears. The determination of the form of meningitis rests upon detection of the inciting organism. The more frequent invaders are the Micrococcus intracellularis meningitidis and the pneumococcus.

EXAMINATION OF THORACIC AND ABDOMINAL FLUIDS.

Technic of Obtaining Fluid.—The point for exploratory puncture of chest or abdomen should be determined only after careful physical examination, and should be below the upper level of the area of dullness. For *paracentesis thoracis* (thoracentesis) the patient should be propped up in bed on pillows or a back-rest. If the point chosen be in the sixth or seventh interspace, it is kept in the midaxillary line, while if it be in the eighth interspace, it should be below the angle of the scapula. *Paracentesis abdominis* is usually made in the median line 5 to 8 cm. above the upper border of the pubic bones. The bladder should be completely emptied, by catheterization if need be, and the patient should sit upright on the side of the bed.

It should be remembered that puncture of the chest is not free from danger, particularly when large quantities of fluid are removed, and the operator should be ready for emergencies. Acute pulmonary edema may occur. Often it may be relieved by the hypodermic injection of morphine and atropine. When these failed, Riesman found dry cupping efficacious.

The skin over the site of puncture should be thoroughly sterilized by scrubbing with green soap, ether, alcohol, and finally by the application of tincture of iodine. Surgical asepsis should be observed with regard to the preparation of the operator's hands and the instruments. Local anesthesia may be effected by infiltrating the skin and subcutaneous tissues with a sterile 1 per cent. solution of novocaine, using a fine hypodermic needle fitted to a glass hypodermic syringe. The needle selected for puncture should be of much larger bore than the ordinary hypodermic needle, since a fine needle would be easily occluded with bits of tissue or clotted fibrin. One of about 18 gauge, fitted with a removable obturator or mandarin, is suitable. If a small quantity is desired for diagnostic purposes only, a glass syringe of the Luer type may be used for aspiration, but if the withdrawal of a large quantity of fluid be indicated, the needle should be one which can be attached to the Potain aspirator.

Examination should be made as soon as possible after its removal. The routine examination should include the following: (1) Observation of gross characteristics—color, odor, turbidity, presence of blood. (2) determination of specific gravity. (3) determination of amount of albumin. (4) cytological study. (5) bacteriological study. (6) (*a*) cover-slip preparations, (*b*) cultures when indicated by other findings and clinical features, (*c*) search for tubercle bacillus.

Gross Characteristics.— *Transudates* are effusions due to disturbances of circulation or to changes in the osmotic relations between blood plasma and body tissues. They are usually transparent, light yellow fluids. *Exudates,* which are the result of inflammatory processes, show considerable variation in appearance, ranging from serous fluids to definitely purulent ones. *Chylous* exudates are white and milky due to the presence of large amounts of fat. They are seen after the rupture of a lymphatic trunk into the pleura or peritoneum. *Chyloid* exudates are seen in malignant involvement of the pleura or peritoneum. They are somewhat milky in appearance though not so much so as are the chylous exudates. The opacity is due partly to fat, largely to degenerated and broken-down cells, and sometimes to lecithin. *Hemorrhagic* exudates are seen after injuries to the chest wall, in the presence of new growths, and when the pleura is involved in a tuberculous process. At times the presence of blood in exudates and transudates is due to trauma inflicted by the exploratory needle. *Purulent* exudates are thicker and yellow to green in color. Leukocytes are present in large numbers; bacteria may be found, and occasionally, crystals of fatty acids, some fat and at times, crystals of cholesterol.

Specific Gravity.—This may be taken at room temperature with an urinary hydrometer. The specific gravity of ascitic fluids and pleural transudates is usually below 1.015 while that of exudates is above 1.018.

Determination of Amount of Albumin.—The fluid should be diluted (adding to one part of fluid 9 parts of distilled water) and thoroughly acidified to litmus with 5 per cent. acetic acid. The Esbach test is carried out as directed in the chapter on Urine (see page 242), multiplying the reading by 10.

Pleural transudates contain less than 25 gm. per liter; peritoneal transudates from 15 to 20 gm. per liter; while pleural exudates contain from 30 to 65 gm. and peritoneal exudates from 20 to 25 gm. per liter.

Cytological Examination.[1]—*Preparation of Sediment.*—The fluid should be examined as soon as possible after its withdrawal. If clotted, the fluid should be shaken in a sterile bottle with small sterile glass beads or stirred with a sterile glass rod in order to cause contraction of the clot and liberation of the entangled cells. The fluid is centrifugalized in a 15 cc. sterile centrifuge tube at a rapid rate (2500 to 3000 revolutions per minute) for from three to five minutes. The supernatant fluid is decanted carefully without disturbing the sediment, which with a platinum loop is mixed with the few remaining drops of fluid and spread upon a slide in constantly increasing concentric circles. The preparation is allowed to dry in the air without the use of heat to avoid distortion of the cell outlines. Treatment with a separate fixative is not necessary, since Wright's stain contains methyl alcohol. Should cover-slips be prepared for bacteriologic examination with other stains, they may be fixed by covering with absolute methyl alcohol for two minutes.

Staining.—Musgrave recommends staining with the following:

Wright's blood stain	3 parts
Absolute methyl alcohol	1 part

The cover-slip is flooded with the stain, which is allowed to remain for a minute or slightly less, when 8 to 10 drops of water are added as in staining blood preparations. The stain, so diluted, is allowed to remain an additional two or three minutes, when it is poured off and the cover-slip flooded several times with water from a medicine dropper and dried by holding between the thumb and forefinger and whisking through the Bunsen flame. It may be mounted in balsam.

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[1] For a discussion of the cytology of serous effusions, see articles by Percy Musgrave.

Findings.—Red blood cells, white blood cells, and endothelial cells may be seen. A differential count should be made of the white cells to determine the proportion of the different forms. Those ordinarily encountered are the *lymphocytes, polymorphonuclear neutrophilic* cells, and occasionally *eosinophilic polymorphonuclears.* Large numbers of broken-down cells may be seen. The *endothelial* cells are cast off from the serous surfaces and are of relatively large size. They may be seen as isolated cells or in groups of two or more. These cells are usually circular in outline with round or oblong nuclei. They are seen in various states of degeneration, sometimes with disintegrated and ragged protoplasm, and sometimes with the protoplasm swollen and more or less vacuolated. They are phagocytic at times, containing partly digested leukocytes. *Cancer cells* may be seen. They are larger than the endothelial cells. Identification, which is not always easy, may be aided by the discovery of nuclear division.

Interpretation of Cytological Findings.—So-called laws regarding the cytology of serous fluids were formulated by Widal and Ravaut. Briefly, these may be summarized as follows:

1. In a tuberculous process the lymphocytes predominate.
2. In an acute infectious process the polymorphonuclear cells predominate.
3. With a mechanical effusion or transudate, endothelial cells are seen in large numbers. They are especially likely to occur in large sheets or plaques.

There are, however, conspicuous and frequent exceptions to these so-called laws. In the early stages of a tuberculous process, polymorphonuclear cells often predominate, while lymphocytosis is sometimes seen in transudates and in infections with staphylococcus, influenza bacillus and pneumococcus. Particularly is this the case in chronic infections.

Bacteriologic Examination.—All purulent fluids and such serous fluids as are not definitely transudates, should be subjected to bacteriological study. Cover-slip preparations may be made of serous fluids as described for cytological study and microscopic examination for bacteria. A preliminary search may be made in the cover-slip stained with Wright's stain, but a Gram's stain should be carried out also. When influenza is suspected, a cover-slip should be stained with dilute carbol-fuchsin. In chest fluids the same varieties of organisms may be expected as are found in the sputum, and the methods given in the chapter on Sputum for staining and cultural work can be adapted to the examination of pleural fluids.

If the *pneumococcus* be present in empyema fluid, suitable media should be inoculated as directed in the chapter on Sputum (see page 362) and the type determined by the agglutination tests. If desired as an additional measure, a portion of the fluid may be centrifugalized at high speed to throw down the pneumococci when the sediment may be taken up with 1 cc. of sterile normal salt solution and injected intraperitoneally into a white mouse, or used for cultural methods.

Influenza bacillus may be cultivated on blood agar, blood oleate agar, or in blood oleate broth (see page 359).

The detection of the *tubercle bacillus* in the pleural fluid is often a matter of the utmost importance. A cover-slip may be prepared as directed for the cytological study, fixed with absolute methyl alcohol, and stained for tubercle bacillus (see page 436). A search conducted in this way, however, is seldom successful.

Inoscopy is the name given by Jousset to a method which he proposed for the detection of the tubercle bacillus. The clot is removed with a sterile wire, placed on a piece of sterile gauze which has been tied across the top of a sterile glass funnel and freed from serum by pouring sterile freshly distilled water through the gauze. It is then picked up with a sterile spatula and placed in a small bottle with about 20 or 30 cc. of the following mixture:

Pepsin	2 gm.
Strong hydrochloric acid	10 cc.
Pure glycerin	10 "
Sodium fluoride	3 gm.
Distilled water	1000 cc.

The bottle containing the clot with the pepsin mixture is placed in the incubator at 37° C. or in a water-bath at 50° C. until digestion has been accomplished. The mixture is then centrifugalized. Cover-slip preparations are made as directed for cytological study and stained for tubercle bacilli. Musgrave states that the majority of the organisms which he found with this method were broader and shorter and some were paler red than those usually found in the sputum.

Animal inoculation is probably the most reliable method for demonstrating the organisms, though the results are delayed. The fluid may be concentrated by centrifugalization (see page 401 for method).

EXAMINATION OF PURULENT EXUDATES.

Pus is usually thick, and yellow or grayish-yellow in color. It contains large numbers of cells, chiefly polymorphonuclear neutrophiles ("pus cells") in various stages of degeneration. The cell outlines are often vague and in many instances nuclei and cytoplasm are poorly stained. Ingested bacteria may be seen within the leukocytes. In gonorrheal pus, eosinophiles are seen.

Pus should be obtained with a sterile swab, platinum loop, or capillary pipette. Smears should be made on slides or cover-slips and stained at once (see page 414 for method). Preliminary examinations may render identification possible on morphology and staining reactions, and at least serve to indicate the type of media which should be employed for cultures.

For routine purposes, Loeffler's methylene blue and Gram's stain should be employed. Gram's stain is most useful in differential diagnosis (see page 436).

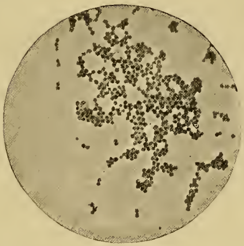

FIG. 122.—Staphylococcus. × 1100 diameters. (Park and Williams.)

Among the organisms which are more frequently found in pathological exudates are staphylococci, streptococci, pneumococci, and gonococci. Organisms of the *staphylococcus group* are characterized by the occurrence in cultures of irregular grape-like clusters (Fig. 122). The cocci are spherical, averaging about 0.8μ in diameter, and are Gram-positive. Cultures are necessary for the differentiation of the varieties.

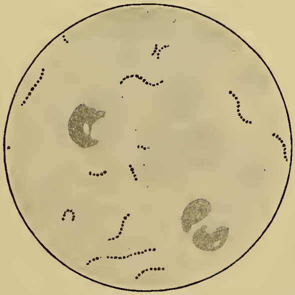

FIG. 123.—Streptococcus in pus. × 800. (Kendall.)

Streptococci may be seen in pus in chains (Fig. 123), but differentiation is not always possible since they may occur as separate cocci and only show chain formation when grown in liquid media. The individual cocci are from 0.5 to 1μ in diameter. While ordinarily spherical, adjacent cocci in chains may show some flattening of the opposed surfaces which are in apposition. Practically all of the streptococci are Gram-positive. Cultures are necessary for the determination of species.

Pneumococci may be seen, especially in pus from pleural exudates. When lance-shaped Gram-positive diplococci are seen, preparations should be stained with one of the capsule stains (see Bacteriological Methods). A description of the organism with methods for cultivation and typing, may be found in the chapter on Examination of Sputum.

The Eye.—Pus from the conjunctiva may show staphylococci, streptococci, pneumococci, diphtheria bacilli and gonococci. The prompt recognition of the latter (see page 393) is a matter of great importance, on account of the rapid and destructive course of the gonorrheal ophthalmia. The pneumococcus is occasionally found associated with a corneal ulcer. The *Koch-Weeks' bacillus* is found in acute epidemic conjunctivitis. It is Gram-negative and similar in morphology to B. influenza, but somewhat longer (Fig. 124). It grows readily upon media containing serum or ascitic fluid and does not require the presence of hemoglobin in the medium as does the influenza bacillus. The *Morax-Axenfeld bacillus* is associated with certain cases of subacute and chronic conjunctivitis in which the inflammation is marked in the angles, particularly about the caruncle. The Gram-negative organisms are short and thick, usually about 2μ long, with distinctly rounded ends, often in short chains. They stain readily with the ordinary stains. They grow only upon alkaline media containing blood or blood serum and when cultivated upon Loeffler's blood serum, cause liquefaction of the medium. The *diphtheria bacillus* may be found in membranous conjunctivitis (Figs. 127 and 128). In examining smears from the conjunctiva, it must be remembered that the Bacillus xerosis is found in normal conjunctivæ and that it cannot be distinguished from the diphtheria bacillus on morphological grounds. Growth upon Loeffler's blood serum is very similar, too, differing only in being a little more delicate. Differentiation can be made only by the use of sugar media. B. xerosis forms acid from saccharose and not

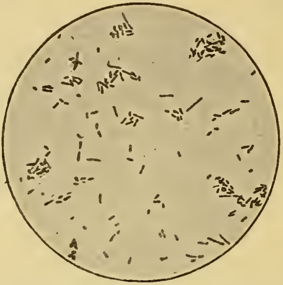

FIG. 124.—Koch-Weeks' bacillus from "pink-eye"—third generation. × 1000 diameters. (Weeks.)

from dextrin while B. diphtheriæ forms acid from dextrin and not from saccharose. The *trachoma bodies* of Halberstädter and Prowazek may be demonstrated by examining scrapings from the conjunctiva. They are small oval or round granules of variable size, typically enclosed in a "mantle;" found within the bodies of the conjunctival epithelial cells, usually near the cell-nucleus. With earlier lesions, the bodies stain a faint blue with Giemsa's stain, while later they stain red. Williams believes that these bodies are degenerated hemoglobinophilic bacilli.

The Ear.—The more frequent causes of acute otitis media are the pneumococcus, the streptococcus, and the diphtheria bacillus. Discharges may occur secondary to other infections, such as typhoid fever or influenza and the exciting cause of the original infection will be found in the discharge. As an otitis becomes chronic, contamination with the staphylococcus may take place. B. pyocyaneus is sometimes found, as is the colon bacillus and Friedländer's bacillus.

The Mouth and Throat.—A large variety of organisms may be found in the mouth. The number depends somewhat upon the care which is given to the teeth. Even in normal individuals, many are always present, including staphylococci, streptococci, occasionally pneumococci, Leptothrix buccalis, and Spirocheta buccalis and Spirocheta dentium. The spirochetes are found in especially large numbers when the teeth and gums are in poor condition. *Leptothrix buccalis* occurs as long, slender-jointed threads. *Spirocheta dentium* is about 4 to 10μ long, is delicate and has deep curves, so that it is easily mistaken for Treponema pallidum. *Spirocheta buccalis* is longer, thicker, and has coarser curves and resembles Spirocheta vincenti. *Endamœba gingivalis* (E. buccalis) is found about the teeth. It may be found in the pus obtained from the sockets of the teeth in cases of pyorrhea alveolaris and has been regarded by some as a causative factor. Ameboid movement may be seen when material is examined at once on a warm stage. The size varies from 6 to 32μ. Ectoplasm is present, the nucleus vesicular and poor in chromatin, and ingested red blood cells, leukocytes, and bacteria are seen. Bass and Johns have suggested the following method of staining. Pus is obtained from pockets about the teeth with a Younger dental scaler No. 22 or No. 23. It may be examined in the moist state on a warm slide, or stained according to the technic of Bass and Johns. The pus is spread to make a thin film, dried in the air, fixed by passing through the flame 2 or 3 times, and covered with a drop or two of carbol-fuchsin. This is washed off immediately with water and replaced with a drop or two of Loeffler's methylene blue, which is allowed to act for about one-half minute. The slide is washed off with water and dried.

Thrush.—When scrapings are taken from the white patches on the surface of the mucous membrane and squeezed out on a slide, a network of *Oïdium albicans* will be seen, containing budding cells which resemble yeast cells and under suitable conditions show mycelia and spores. There are two varieties, one with small spores which does not liquefy gelatine and one with large spores which does liquefy gelatine. Sugars are fermented.

Pseudomembranous lesions of the mouth, tonsils, and pharynx are of great importance from a clinical standpoint. Given a membranous lesion, the principle possibilities are diphtheria, Vincent's angina, and syphilis.

Syphilitic Lesions.—A primary lesion of the mouth or tonsil is occasionally seen. There is usually much induration of the deeper tissue. The Treponema pallidum (Fig. 125) may be found though there is some possibility of confusion on account of the presence of Spirocheta dentium in the mouth. In the secondary stage, the patches are usually flat, and do not show a distinct membrane, only a filmy, gray surface. The likelihood of confusion with diphtheria or Vincent's angina is slight on account of the duration of the lesions, their number, and appearance. In this stage the Tr. pallidum may be demonstrated and the Wassermann reaction is almost invariably positive. In the so-called tertiary stage, there may be lesions of varying size, oftentimes of great extent, showing marked loss of tissue, and covered at times with a dirty gray or yellow pseudomembrane. The history of chronicity, admission by the patient of infection and a positive Wassermann reaction will aid the diagnosis. Tr. pallidum is rarely found in the tertiary stage.

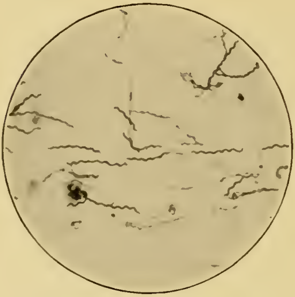

FIG. 125.—Treponema pallidum. (Kendall.)

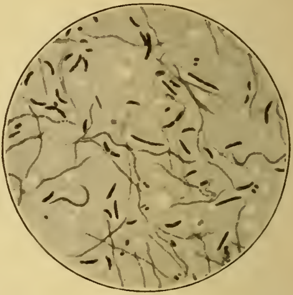

FIG. 126.—Vincent's angina. Bacillus and Spirillum fusiformis. (Kendall.)

In Vincent's angina, the lesion usually involves the tonsils and often the soft palate. A punched-out ulcer results and tissue destruction may cause the loss of tonsils, uvula, and portions of the palate. The ulcerated area is covered with an adherent dirty gray or yellow membrane which is detached with some difficulty. After cleaning the surface by gentle swabbing, portions may be removed for examination with a dull curette, placed on a slide, and squeezed with the aid of a second slide to make a thin preparation. A clear picture is given by staining for five minutes with diluted carbol-fuchsin (one part of carbol-fuchsin and 9 parts of distilled water). Two organisms are seen, the Spirocheta vincenti and B. fusiformis. The spirochetes are motile, rather slender, feebly staining organisms, about 10 to 20μ long, and irregularly curved with shallow undulations. The fusiform bacillus is from 3 to 10μ long, and thicker at the center than at the end, which taper to a point. The rods stain rather unevenly so that they appear to be barred (Fig. 126). The bacillus is not motile, though Stitt states that he has observed feeble motility in specimens mounted in saliva. Both organisms are Gram-negative, though there is a tendency to some variation on the part of the bacillus. It should be remembered that these organisms may be secondary invaders, and that they are frequently present in great numbers in syphilitic lesions.

In *diphtheria*, the membranous exudate is usually seen in the nasopharynx, on the tonsil, and less frequently in the nose and trachea. Local treatment should be withheld for twelve hours previous to making cultures. In making cultures from children, the wire bearing the cotton swab should be curved like a laryngeal applicator and introduced deep into the throat. Inoculation should be made on Loeffler's blood-serum; then the swab should be rubbed on slides and the resulting smear stained immediately.

Cultures are incubated at 37° C. for not over sixteen or eighteen hours, when some of the growth should be removed with a sterile platinum loop and transferred to a slide. A thin emulsion should be made with a droplet of sterile water. This is dried, fixed in the usual way, and stained by one of the following methods:

1. Stain with Loeffler's methylene blue for five minutes, using no heat. Pour off the stain and wash with repeated changes of 0.5 per cent. acetic acid until the preparation assumes a gray tint, showing only a blue tinge. This method has the advantage of simplicity and gives a very clear-cut picture. The polar granules are dark blue, standing out clearly in contrast in the very pale bodies of the bacilli.
2. Stain with Neisser's stain for from one to three seconds. (See page 436.) Wash in water and stain for from three to five seconds with Bismarck-brown solution. Wash with water and examine with oil-immersion lens. With this method the bacilli are seen as brown rods containing bluish-black granules.

Diphtheria bacilli are straight or slightly curved rods, staining irregularly, showing metachromatic granules which are spoken of as "polar granules" when situated at the ends of the rods. Barred forms appear (Figs. 127 and 128). A tendency to parallel arrangement is seen. Oftentimes the rods are quite irregular and clubbed ends are seen. The morphology of the organism varies at different periods of development. This is to be remembered in examining cultures, and makes the time of incubation a matter of practical importance. Various workers have divided the organism in groups based on the morphology.

There is no way in which virulent strains can be distinguished from non-virulent ones on morphological grounds. One cc. of a forty-eight-hour broth culture may be injected intraperitoneally into each of two guinea-pigs. If the culture be virulent, death should occur in one to two days. At the point of inoculation there will be a sero-sanguineous exudate.

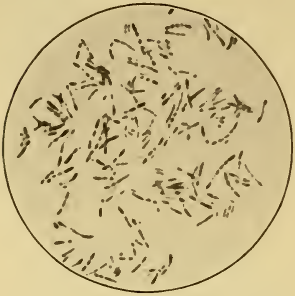

FIG. 127.—Bacillus diphtheriæ, methylene blue stain. × 1000. (Kendall.)

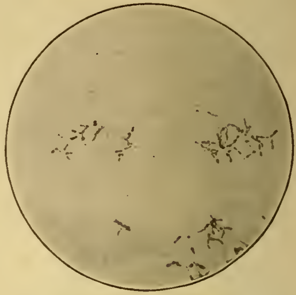

FIG. 128.—Bacillus diphtheriæ, branching. × 800. (Kendall.)

The Nose.—Cultures from the nose are of importance at times. They may be made by introducing a swab into the nares. A speculum may be used but it is not usually necessary. For cultures from the retropharyngeal space, a special tube has been devised by West. This consists of a glass tube about 7 mm. inside diameter and about 16.5 cc. long, curved at one end nearly to a right angle. Inside the glass tube is a copper wire carrying at one end a cotton swab. The swab is pulled back into the tube, both ends of the tube are plugged, and the apparatus is sterilized. When cultures are to be made, the glass tube is carried back over the tongue as near to the posterior pharyngeal wall as is possible. The copper wire is pushed forward enough to permit swabbing of the mucous membrane, when it is pulled back into the glass carrier and removed from the mouth. The plate upon which cultures are to be made is then swabbed with the material which has been obtained. Many workers prefer simply a curved wire the distal end of which has been wrapped with cotton to form a swab.

Nasal cultures assume especial importance in the detection of carriers of meningococci, streptococci, and diphtheria bacilli. In attempting to determine carriers, suitable media should be used, for example, blood-agar plates for the streptococci, and glucose agar (0.4 + to phenolphthalein) with either blood, blood serum, or ascitic fluid for meningococci, and Loeffler's blood serum for diphtheria bacilli.

Urethra and Vagina.—Pus is obtained with a sterile swab. With the adult female, a specimen should be taken from the mouth of the urethra, and another from the mouth of the cervix with the aid of a speculum. The material is spread upon the slide gently to avoid injuring the cells and fixed and stained with Gram's stain and methylene blue according to the methods described in the chapter on bacteriological technic (see pages 435 and 436).

Gonococci are seen as Gram-negative, flattened, biscuit-shaped spheres, occurring in pairs with flattened sides in apposition. The cocci are about 0.8μ wide by about 1.6μ long. Usually a number are clustered together, and in acute cases many are found within the cytoplasm of the leukocytes (Fig. 129). They do not grow well on ordinary culture media, requiring the presence in medium of blood serum, ascitic or hydrocele fluid. In pus from the male urethra the occurrence of organisms with the morphology and staining reactions just described is ordinarily taken as sufficient for the purpose of practical diagnosis. An organism which may occur in the urethra and which may cause confusion is *Micrococcus catarrhalis,* which is also a Gram-negative diplococcus. In contrast to the gonococcus this grows readily upon ordinary media. "Shreds" may be found in the urine in acute and chronic gonorrheal infections (see page 208).

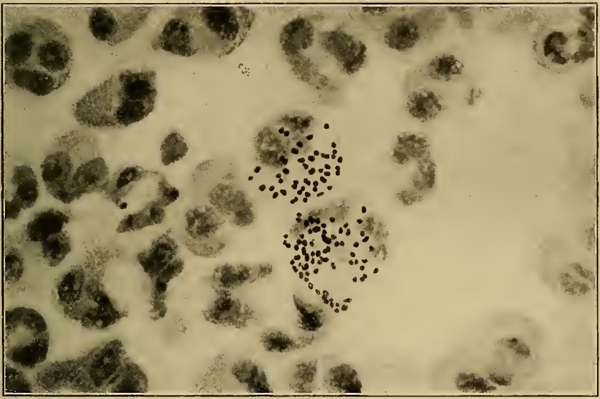

FIG. 129.—Gonococcus smear of pus from acute case. Methylene blue stain. (Warden.)

SKIN INFECTIONS.

A number of chronic infections of the skin are caused by the pathogenic moulds. Examination of scrapings is of diagnostic assistance. The crust, skin, or an infected hair is removed and softened with a drop or two of 20 per cent. sodium hydrate, is pressed out on a slide with a cover-slip, when the slide may be heated slightly over the flame. Staining is usually unnecessary, but may be accomplished by placing some of the material on a slide. It is smeared with a drop of serum or Mayer's egg-albumen and treated with alcohol and then with ether to free it from fat. It may be stained with Wright's stain or by Gram's method.

Achorion Schoenleinii.—Favus is a chronic disease, usually attacking the hairy portions of the body. Small yellow sulphur-like cupped-out crusts are seen surrounding a hair or hairs. When removed and treated with sodium hydrate, the microscopic examination shows that the crust (scutulum) is made up of desquamated epithelial cells, dried serum, and double-contoured spores of irregular shape, lying in a net-work of mycelial threads, the fungus being known as Achorion Schoenleinii. It is possible at times to see filaments of mycelia which give off hyphæ terminating in small knobs. Cultures may be obtained by grinding some of the material in a sterile mortar with fine sterile sand. The triturated mass may be inoculated into melted agar and plates poured. Growth is best, however, on the surface of Sabouraud's glucose or maltose agar and appears in from six to nine days in the form of a pale yellow whorl the surface of which takes on a convoluted appearance as the colony increases in size. To inoculate, the tip of a curved pointed platinum needle is moistened on the sterile medium. A small particle of the scutulum is picked up on the moistened end and deposited on the medium. Several different plantings should be made on each tube. In making cultures of hairs in a case of favus, it is not necessary to choose any particular hair. A hair may be removed from the scalp, cut into small pieces with a sterile knife blade on a sterile glass slide and planted on the medium, using the same technic as for favus cultures.

Ringworm.—Ringworm of the hairy portions of the body (Tinea tonsurans, T. barbæ, or Tinea sycosis) and of the hairless portions of the skin (Tinea circinata) is caused by several varieties of trichophyton which belong to a group of fungi imperfecti. There are two types of fungus, those with small spores (about 2 to 3μ in diameter) (Trichophyton microsporon) and those with larger spores (about 7 to 8μ in diameter) (Trichophyton megalosporon). The small-spored variety is found throughout the hair substance, from which branches pass out to the surface. Ectospores are found on the surface in zoöglea-like masses. The mycelium is difficult to see. The microsporon is the common fungus in Trichophytosis capitis as seen in America and England. With Trichophyton megalosporon endothrix the mycelium occupies the center of the hair-shaft, running its entire length while with Trichophyton megalosporon ectothrix, the fungus is found in the intrafollicular region and forms a sheath between the hair and the follicle. The fungi are seen as finely interlaced separate threads upon which swellings appear. From these the chlamydospores develop. Ringworm of the body may be caused by either the small spore or large spore organism.

These fungi are best cultivated upon media enriched with glucose or maltose. Infected hairs or scrapings of skin may be treated for two hours with 60 per cent. alcohol to kill the bacteria before inoculation and then planted according to the technic given for favus cultures. In this condition, in distinction from favus, all the hairs are not affected and one must select the broken off stubs which show just above the surface of the skin and which on removal are seen to have a glove-like whitish deposit over the outside, *i. e.*, a fungus growth. Sabouraud's medium is generally recommended:[1]

Maltose or glucose	4.0 gm.
Peptone	1.0 "
Agar	1.8 "
Water	100.0 cc.

Trichophytosis Unguis.—In making cultures from ringworms of the nails the same technic may be employed as with the cultures of the hairs, cutting the pieces finely. For microscopic examination, it is often necessary to digest the broken off nails ten or twelve hours in a 20 per cent. solution of sodium hydrate before making the examination.

Microsporon Furfur.—This fungus is the cause of tinea versicolor (pityriasis versicolor), in which the skin shows dirty yellow or brown patches. The short, thick hyphæ lie in a thick net-work and are usually not jointed or branched. The spores (conidia) may be seen at the extremities of the hyphæ. The spore, whose diameter is a little greater than that of a red blood cell, contains a large nucleus. The fungus is cultivated with great difficulty.

Oïdium Albicans (*Monilia albicans*).—In blastomycosis of the skin there is a nodular eruption which later forms a pustule. This discharge shows oval yeast bodies, about 12 to 14μ in diameter. Budding is seen. Cultivation is usually carried out easily by inoculating pus on Sabouraud's glucose or maltose agar, which inhibits the growth of the ordinary cocci. Ormsby advises making cultures from the minute, deep-seated abscesses.

Sporotrichosis.—In sporotrichosis of the skin there is a more or less nodular eruption which may or may not be generalized, and from which profuse yellow pus may be obtained. The process is chronic, tending to spread. Cultures from the pus on Sabouraud's media within four or five days show a beginning growth of dark brownish-black colonies which assume a more or less convoluted form and grow rapidly. The surface is quite characteristic, resembling the skin of a rattle-snake in color.

Infestation with Itch Mite and Insects.—*Sarcoptes Scabiei.*—The itch mite is found on those portions of the body covered with thin skin, particularly between the fingers, and in the bend of the elbow- and knee-joints, in the inguinal and mammary region, and on the penis. The body is nearly circular in outline, marked by transverse striæ on the back. There are four pairs of legs, two anterior and two posterior. The mite burrows under the skin in somewhat tortuous tunnels, which may be from a few millimeters to a centimeter in length. At the end of the tunnel the female may be found. Eggs and fecal material are deposited in the tunnels. The males are seldom found since they die after copulation. To assist in diagnosis, the tunnel may be opened with a needle and the contents pressed out upon a slide for examination.

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[1] In culture work with ringworm, it is found that the characteristics vary with media from different sources. Hence, Sabouraud has recommended the use of the media first employed by him in his work, which is now used universally by men doing this work. The material can be procured from E. Cogit & Co., Boulevard St. Michael, Paris, France. The maltose and glucose, while in crude form, are much cheaper than the purified products and serve the purpose. In ordering it is well to ask for the preparations used by Sabouraud in his work.

Pediculus Capitis.—The head louse lives in the hair of the scalp, rarely in the hair of other portions of the body. The color varies from light gray to black. The thorax and abdomen are of the same breadth, the former having six segments, the latter three segments and three pairs of legs. The male is from 1 to 1.5 mm. long and the female from 1.8 to 2 mm. long. The ova (nits) are pear- or barrel-shaped and are attached to the shaft of the host's hair with a clasping collar.

Pediculus Vestimenti.—The body louse lives on the clothing around the neck, throat and trunk. It is 2 to 4 mm. long and grayish-white in color. The abdomen is broader than the thorax and the antennæ are longer than those of the head louse.

Pediculus Inguinalis.—The "crab-louse" is found in the hair of the inguinal region, rarely on the head, but occasionally on other parts of the body. The males are from 0.8 to 1 mm. long and the females about 1.2 mm. long. The abdomen has nine segments.

The diagnosis in cases of pediculosis may be made by finding the adult insects, but more readily by finding the eggs attached to the hairs in the affected regions.

DETECTION OF TREPONEMA PALLIDUM IN SYPHILITIC LESIONS.

In an early chancre, the organisms are usually demonstrated readily. After healing has commenced or after antiseptics or caustics have been used, the search may be fruitless. For this reason, local treatment should be withheld until a diagnosis has been made. The organisms may be found in any of the secondary lesions. In material obtained from the buccal cavity, there is always possibility of confusion on account of the presence of Spirocheta dentium. Treponemata are seldom found in serum obtained from tertiary lesions and rarely even in suitably stained sections of tissue. An essential part of the technic is obtaining "irritation serum." At times, serum wells out freely without irritation, but such cases are exceptional. The treponemata may be demonstrated in stained specimens, or in unstained specimens with the aid of dark-field illumination. The latter is distinctly the method of choice since movement may be studied as well as form, and the organisms are found much more readily. All staining methods are open to the objection that the apparent width of the organism varies with the thickness of the film.

OBTAINING IRRITATION SERUM FOR EXAMINATION.

The lesion should be thoroughly cleaned by repeated applications of sterile physiological salt solution and the surface scraped with a dull curette until the blood begins to ooze. Suction should be applied. A piece of apparatus may be made from a piece of stout glass tubing with inside diameter 18 to 20 mm. with one end drawn out to fit a small rubber bulb. (Bulbs sold by stationers for cleaning fountain pens are convenient). This is removed when the blood or serum flows freely. The irritation serum which exudes after blood flow ceases is transferred to a clean cover-slip with a fine capillary pipette or a sterile platinum loop. The serum should be allowed to dry when a stain is employed, but when the dark-field illuminator is used, the moist preparation is diluted with a drop of normal salt solution, mounted and examined immediately.

Examination with the Dark-field Illuminator.—Two types of condensers are furnished by manufacturers, one of which is held on the microscope stage with clips, while the other is substituted for the Abbé condenser. Directions for setting up the illuminator are furnished by the manufacturers. An intense light, such as that from a small arc lamp, is reflected from the flat side of the mirror. The entire oil-immersion lens is removed from the brass collar and screwed onto a similar collar supplied with the condenser, fitted with a funnel stop to diminish the intensity of the light. The remounted lens is then placed on the microscope and used for the examination. Oil is placed on the center of the condenser, the light adjusted so that the microscopic field is illuminated properly and the draw tube of the microscope pulled out to the proper length. Object slides should be prepared for mounting the serum, by making in the center circles with cedar oil about 1 cm. in diameter. The apparatus should be prepared before obtaining serum.

Irritation serum is placed on cover-slips as directed, the cover-slips are turned over and mounted on the slides so that the serum is enclosed between the slide and cover-slip in a ring of cedar oil. A drop of cedar oil is put on the cover-slip and the slide then placed on the dark-field illuminator. When the slide has been made properly, the light rays pass from the condenser successively through oil, slide, irritation serum, cover-slip, oil, and finally the lens.

While focussing, great care must be taken not to force the lens through the cover-glass. It is better to watch from the side while lowering the lens into the oil, then to move the lens upward with the eye at the ocular. The treponema pallidum[1] appears in a dark field as a white, tightly curled organism swimming actively with corkscrew motion. The coils, which are close and regular, number from 6 to 26. The length is from 10 to 20μ, and can be estimated in preparations by comparison with the diameter of the red blood corpuscles (average diameter 7μ) (Fig. 130).

Giemsa Stain.—The irritation serum is allowed to dry in the air and is fixed by flooding the cover-slip with absolute methyl alcohol which is allowed to remain for two or three minutes when it is whisked off and the cover-slip dried in the air.

The stain is prepared by mixing 10 drops of Giemsa stain, 1 drop of 1 per cent. potassium carbonate solution and 10 cc. of distilled water. This is placed in a Stender dish. The cover-slip is carefully floated, film side down, on the surface of the stain. This may be done by lowering the cover-slip on a piece of fine wire bent to form a wide loop. The dish is covered and the cover-slip allowed to stand for twenty-four hours, when it is removed, washed carefully with distilled water and dried in the air without blotting. Mounted in balsam, it serves as a permanent preparation. The treponemata stain deep pink.

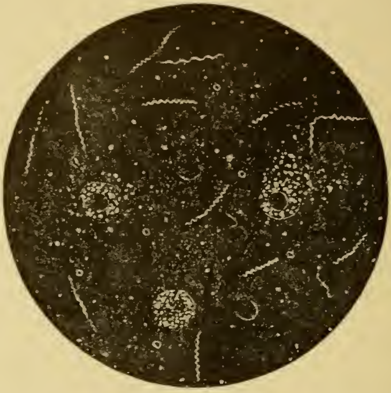

FIG. 130.—Treponema pallidum appearing as bright refractive body on a dark fluid as shown by India ink or ultramicroscope. (Park and Williams.)

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[1] The terminology of the organism has been a moot question and even now there is no real agreement. A discussion of the nomenclature is given by Clifford Dobell. The organism was discovered by Schaudinn, who called it Spirocheta pallida. Its specific name is then properly pallida, Schaudinn, 1905. The generic name Spirochætæ was not correct, however, and several others have been proposed, including Spironema, Treponema, and Microspironema. Since the name Spironema has priority in time over the others, it is held by Dobell that it must be accepted as correct unless it can be shown to be unavailable. The opinion of systematists, he states, is that the spirochætes, including that of syphilis, are not related to the Protozoa, as the name Treponema implies, but to the Bacteria, and that the name Treponema is not justifiable.

Wright's Stain.—Wright's stain may be employed. (See page 440). Staining should be allowed to proceed longer than with blood, for ten or even fifteen minutes.

India-ink Method.—In Burri's method a drop of irritation-serum is mixed with a small drop of India-ink at one end of an object-slide. The mixture is spread on the slide with a second slide (see page 451).

A good grade of India-ink should be employed, since the method has been criticized on account of artefacts, presumably fibers of some sort, which resemble treponemata. The smear which should be dark-brown in color, is allowed to dry and is examined with an oil-immersion lens. The organisms appear as light spirals on a dark-ground.

DETECTION OF ORGANISMS IN SOFT CHANCRE.

There is no method of making a diagnosis between the so-called "soft chancre" of non-specific nature and the "hard chancre" of syphilis except by finding the Treponema pallidum. The names are inappropriate since many so-called "soft chancres" are found to harbor treponemata. Syphilis cannot be excluded simply because no treponemata are found on one or two occasions, for their absence may be due to a number of factors, such as local treatment, formation of scar tissue, or faulty technic.

The bacillus of Ducrey has been found frequently in soft chancres. It is a Gram-negative small bacillus measuring about 0.5 by 1 to 2μ, occurring in chains of considerable length, and growing on blood agar.

A safe clinical rule is, to make the diagnosis of a non-syphilitic lesion only after syphilis has been excluded by clinical observation supplemented by repeated laboratory examinations (search for the treponemata and Wassermann reactions on the blood), extending over a period of at least twelve weeks.

It has been shown recently that many of the "soft sores" are accompanied by the spirochete and fusiform bacillus identical with or similar to those found in Vincent's angina. Whether or not these stand in the relation of causative factors in certain cases is not known, but their recognition is a matter of some importance, since suitable treatment often results in prompt healing. After cleaning the lesion with physiological salt solution, some of the membranous material may be removed with a dull curette, spread between two slides, and stained as directed for the organisms of Vincent's angina, or the material may be mixed with a drop of sterile normal salt solution and examined with the dark-field illuminator.

EXAMINATION OF SEMEN.

Spermatozoa.—Examination of semen is sometimes required in cases of sterility. A thermos bottle should be heated by filling it with water somewhat above blood heat. This is poured out, and the condom containing the semen placed in the bottle. The specimen should be examined without unnecessary delay. A drop of the material is placed on a warm slide and covered with a cover-slip. Normally, actively motile spermatozoa should be seen in considerable numbers. They are about 45 to 50μ in length with an oval head followed by a cylindrical middle piece tapering to form a long tail which shows whip-like motion in fresh specimens.

In the semen there are also found epithelial cells, corpora amylacea, and lecithin globules. The latter give semen its milky appearance. Under abnormal conditions, red and white blood corpuscles may be seen.

Florence Reaction.—To identify stains due to semen, the Florence reaction may be used. The reagent consists of iodine, 2.54; potassium iodide, 1.65; distilled water, to 100 cc. The iodine should be washed with distilled water before the mixture is made. The stain is scraped or cut off from the material on which it is found and placed on an object slide. After adding a few drops of the reagent, the mixture is covered with a cover-slip and examined promptly under the miscroscope. Dark brown crystals are formed. These are lance-shaped or rhombic in form and may be grouped to form rosettes. The reaction is given not only by human and animal semen but also by various organ extracts, extracts from seeds, etc. It is evident therefore that while a negative result has some significance, a positive one has none.

EXAMINATION OF BRAIN TISSUE FOR NEGRI BODIES.

It has been found that Negri bodies may be found in the nervous system of almost all animals suffering with rabies. These are found more readily in the large ganglion cells of the hippocampus major. The bodies are regarded as of protozoal origin. Their detection is of great clinical importance since unfortunately it often happens that a presumably rabid dog is killed before the development of symptoms which would permit the making of a diagnosis. In such cases, the dog's brain should be examined for Negri bodies, so that when they are present, the individual who has been bitten may be given Pasteur treatment at once.

The animal's cranium is opened and a small bit of brain substance removed from (1) the convolution around the crucial sulcus; (2) the gray matter of the cerebellum; (3) Ammon's horn. Each particle is covered with a cover-glass which is pressed down to flatten out the tissue and then dragged along the slide so that the cells will be separated. Slides may be made by the "impression method," touching the brain tissue to slide in a number of places so that a few cells are left in each area. After the tissue has dried, it is fixed for ten seconds with neutral methyl alcohol to which 0.1 per cent. picric acid has been added. This is blotted off gently and the slide covered with William's modification of Van Giesen's stain:

Saturated alcoholic solution of fuchsin	5 cc.
Saturated alcoholic solution of methylene blue	10 "
Distilled water	30 "

The stain keeps a long time in the refrigerator.

The stain is heated until it steams and is washed with water, and the slide is examined with the oil-immersion lens. The Negri bodies are seen within the ganglion cells. Their size is variable, ranging from 1 to 3μ in diameter up to 6μ in width by 27μ in length. The smaller forms are seen in animals killed with "fixed virus," and the larger ones in animals with "street virus." One or more nuclei are present. The bodies stain magenta, the red blood cells yellow or salmon color and the body of the nerve cell blue. Animal inoculation may be carried out as a confirmatory test. A salt-solution emulsion is made of the brain or spinal cord of the infected animal and injected through a trephined opening in the skull of a rabbit. Since diagnosis was delayed fifteen to twenty days, Wilson recommended the use of guinea-pigs, which develop symptoms much more quickly.

ANIMAL INOCULATION.

The technic of mouse inoculation for pneumococcus typing has been described (page 363). The technic for intravenous injection of rabbits has been given in the portion of the chapter on the Examination of the Blood which deals with immunological methods. For the detection of small numbers of tubercle bacilli, a guinea-pig is inoculated with some of the infectious material. Small young animals should be selected. When the material is fluid and a sediment can be obtained, centrifugalization should be employed in order to secure concentration, and the sediment should be injected. When the material is free from contamination and of such a nature that few tubercle bacilli are to be expected (as in the case of pleural fluids), about 10 cc. may be injected intraperitoneally. If, on the other hand, the material be purulent, a much smaller quantity should be injected subcutaneously in the inguinal region. If it be necessary to inject contaminated fluid intraperitoneally, as may be the case with urine, a number of animals should be employed. At least two pigs should be injected.

Should the animal be living at the end of six to eight weeks, it should be killed. After death, the animal is tied down on a small board on its back with extremities drawn out with cords to the four corners of the board. The fur is plucked out in the median line from the sternum to the pubis and the skin soaked with alcohol. If cultures be made, the line of incision should be burned with a hot instrument and sterile instruments should be used, but if merely an inspection for tubercles be desired, these precautions are unnecessary. The peritoneum is cut open, the intestines are removed *in toto*, and search is made for gray tubercles or for yellowish-gray cheesy lymph nodes. Suspicious tissue may be removed, transferred to a slide, crushed with another slide, and stained for tubercle bacilli.

EXAMINATION OF MILK.

Human Milk.—The examination of the milk of nursing mothers is sometimes desirable in order to determine its suitability for infant feeding. At least 10 cc. of milk should be obtained with the breast pump. Examination may be conducted with the aid of Holt's apparatus, consisting of a small hydrometer (Fig. 131, *A*) and a cream gauge, (Fig. 131, *B*) which is in reality a 10 cc. glass-stoppered cylinder graduated in 0.1 cc. The *reaction* should be taken with litmus paper and the *specific gravity* with the hydrometer as is the specific gravity of urine.

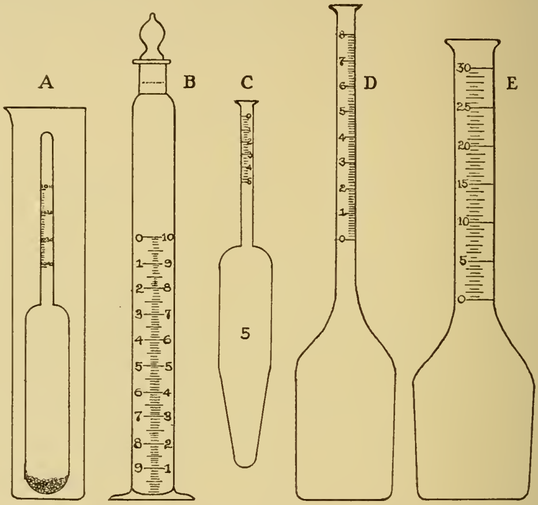

FIG. 131.—Glassware for milk testing. *A*, hydrometer; *B*, Holt's cylinder; *C*, centrifuge tube for human milk; *D*, bottle for milk testing; *E*, bottle for cream testing.

For fat determinations the 10 cc. cylinder is filled with milk to the 10 cc. mark and allowed to stand at room temperature. At the end of twenty-four hours, the cream will have risen to the top. The percentage is read. The fat may be determined from this by multiplying by 0.6, since it constitutes 60 per cent. of cream.

A modification of the Babcock method may be used for the determination of the fat content. A special centrifuge tube is necessary (Fig. 131, *C*). This fits into the shield of the ordinary medical centrifuge. Five cc. of milk are placed in the tube with a pipette, and 1 cc. of a mixture of equal parts of amyl alcohol and hydrochloric acid is added. (While this mixture may keep for several weeks, it is not suitable for use after it has turned dark.) Sulphuric acid (specific gravity, 1.83) is added cautiously to the zero mark (see directions for Babcock test on dairy milk) and the tube is centrifugalized for about three minutes at a rate of 1000 revolutions per minute or until the fat has risen to the top of the tube. The amount may be read on the scale, each division representing 0.2 per cent.

Normally, there is 7 per cent. of cream or 4.2 per cent. of fat. The specific gravity is from 1.028 to 1.032. A diminution in specific gravity may be due either to an increase in the fat content or an increase in the amount of total solids.

The *protein content* may be determined by placing the milk in a small separatory funnel in the incubator for twenty-four hours. At the end of this time, the cream will have risen to the top and the thin liquid below, containing protein and lactose, may be drawn off. After cooling, it is diluted with 9 parts of water and used for the Esbach test, which is carried out as directed for urine. There is about 1.5 per cent. of protein in normal milk, although the amount is much higher immediately after delivery.

For the determination of lactose, milk should be acidified with acetic acid, boiled, and filtered to remove the coagulated proteins. The filtrate is used for the determination of sugar by Benedict's method, preferably using Myers' technic on account of its adaptability to small quantities (page 245). Ten cc. of Benedict's solution are reduced by 0.027 gm. of lactose. Normally, there is 7 per cent. of lactose in human milk. The amount is less immediately after the birth of the child.

Dairy Milk.—Dairy milk is occasionally sent to the clinical laboratory for examination. The specimen should be thoroughly mixed to ensure even distribution of the cream, by pouring the milk from the bottle into a large beaker and back into the bottle several times. Should the milk come in a can, it should be poured back and forth from one can into another. For determination of fat content, the Babcock test is usually employed. This requires the use of special bottles which may be centrifugalized in special shields in a heavy centrifuge. Shields may be obtained to fit the large head of the centrifuge recommended for immunological work. Place 17.6 cc. of milk in the bottle with a special pipette (Fig. 131, *D*), holding the bottle in a slightly tilted position with the tip of the pipette into the neck, leaving an exit for the air in the bottle. None of the milk should be lost in the process of transfer. Add 17.5 cc. sulphuric acid (specific gravity between 1.82 and 1.83) from a cylinder or pipette, again holding the bottle at an angle and allowing the acid to flow down the wall. The acid must be run into the center of the milk as a fine stream. After the procedure has been properly carried out, there should be two distinct layers with only a slight band of charred milk between them. The layers should be mixed by a combined rotary and shaking motion. Curds should not be allowed to get into the neck of the bottle. The process of mixing should not be interrupted until the curds which form at first have been completely separated. The mixture turns dark brown and becomes hot due to the action of the acid. The bottles are centrifugalized about five minutes at a rate of 600 to 1200 revolutions per minute, or until all the fat has been brought to the surface of the liquid. It may be necessary to fill the centrifuge shields with hot water to keep the fat liquid. When separation is complete, hot water is added with a pipette to bring the upper level of the fluid to a point near the graduations on the neck. The bottle is centrifugalized for one minute, hot water added again so that the lower level of the fat will come within the graduated scale, and the bottle centrifugalized for a minute. The amount of fat may be read on the scale. It is convenient to use a pair of dividers, measuring from the *top* of the fat column to lower line of separation between fat and water. It is important to note that the reading is taken at the top of the meniscus. The dividers may then be moved to a convenient place on the scale and the amount of fat read. Each division of the scale represents 0.2 per cent.

Different types of bottles should be used for testing skimmed milk and cream. The bottle suitable for skimmed milk has a neck with smaller internal diameter and the divisions of the scale represent one-twentieth of 1 per cent. The bottles for cream have a much wider neck and the scales read up to 30 per cent. (Fig. 131, *E*). In testing cream, 18 cc. instead of 17.6 cc. should be used, on account of the lower specific gravity.

The specific gravity of the acid may be determined by weight. A test bottle is dried, weighed, filled to the zero mark with sulphuric acid and weighed again. The difference between the two weights represents the weight of acid. The acid is poured out and the bottle rinsed with several changes of water until the water fails to redden blue litmus. Distilled water is poured in to the zero mark and the bottle weighed again. From this weight is subtracted the weight of the empty bottle to obtain the weight of water. The specific gravity of acid is obtained by dividing the weight of the acid by the weight of the water. The quotient should fall between 1.82 and 1.83. Care should be taken to dry the outside of the bottle each time.

Tests for Preservatives.—Formaldehyde.—The tests for formaldehyde are really general tests for the aldehyde group: (1) Three cc. of milk are diluted with an equal quantity of water, and the mixture is placed in a test-tube. Sulphuric acid (specific gravity, 1.82–1.84) is allowed to run from a pipette to form a layer under the milk, just as nitric acid is run in when performing the nitric acid test for albumin in urine. If formaldehyde be present, a violet ring is formed at the junction of the two liquids. This color reaction may be noticed in carrying out the Babcock test. (2) Five cc. of milk are placed in a white porcelain evaporating dish with an equal quantity of water. To this are added 10 cc. of hydrochloric acid and a few drops of 10 per cent. ferric chloride. A violet color is seen in the presence of formaldehyde.

Boric Acid.—Ten cc. of milk are alkalinized with 10 per cent. sodium hydrate and evaporated. The residue is incinerated and the ash moistened with enough very dilute hydrochloric acid to give it a distinctly acid reaction. A piece of turmeric paper is moistened with the solution. The paper is then dried on a watch-glass placed over a boiling water-bath. When boracic acid or borates are present, the paper turns reddish-brown on drying.

Bacteriologic Examination of Milk.—The specimen should be thoroughly mixed as directed for the Babcock test, using vessels which have been carefully cleaned. The milk is diluted by adding 1 cc. to 100 cc. of sterile bouillon. Sterile pipettes should be used for measuring these quantities. Each cc. of the dilution contains 0.01 cc. of milk.

Culture tubes containing nutrient agar should be placed in a water-bath with boiling water to liquefy the media. The temperature of the water in the bath should be reduced to 43° C. Some of the milk dilution is added to each tube, 1 cc. to one tube (*A*), 0.1 cc. to a second (*B*), and 0.01 cc. to a third tube (*C*). When there is reason to believe that the milk is unusually dirty, 1 cc. of the 1 : 100 dilution may be added to another 99 cc. of sterile bouillon. This will give a 1 : 10,000 dilution, 0.1 cc. of which may be added to a fourth tube (*D*) The medium and the milk in each tube are thoroughly mixed by rolling the inoculated tubes between the palms of the hands, keeping the tubes upright. The mouths are then passed through the flame and the contents of each tube poured into a Petri dish. The plates are covered and, after the medium has solidified, are inverted and placed in the incubator at 37° C. At the end of forty-eight hours, the number of colonies is counted. A plate should be selected for counting which contains less than 100 colonies. A ruled plate counter is convenient. In estimating the number of bacteria, the number of colonies is multiplied by the dilution. The following factors may be employed when the dilutions are made according to the method which has been given: Tube *A*, 100; Tube *B*, 1000; Tube *C*, 10,000; Tube *D*, 100,000. For example, 20 colonies on the plate made from Tube *B* means that there are 20,000 bacteria in 1 cc. of milk.

CARBON-DIOXIDE TENSION OF THE ALVEOLAR AIR.

The significance of reduction in the carbon-dioxide tension of the alveolar air has been referred to in the discussion of tests for acidosis in the blood (see pages 198 and 211). The tension is dependent upon the CO_2 content of the blood and the findings follow those which are obtained in determining the CO_2 combining power of the blood.

Fridericia Method.—*Apparatus and Reagents Required.*[1]—(1) Fridericia apparatus (Fig. 132). There should be two stop-cocks, the upper one an ordinary two-way cock and the lower one a three-way cock of wide bore (10 mm.). The volume should be 100 cc. If not exactly 100 cc., then the scale should be graduated in percentages of the actual volume up to 8 per cent.; (2) rubber bulb to fit outlet; (3) water-bath or pail in which the Fridericia apparatus may be stood upright; (4) sodium hydrate, 20 per cent. solution; (5) dilute acetic acid.

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[1] An accessible description is given by Poulton.

Principle.—The alveolar air is exhaled and 100 cc. is caught and retained in the portion of the apparatus between stop-cocks *A* and *B*. Sodium hydrate is drawn in to absorb CO_2. The amount of the gas absorbed is measured by the difference in volume of the alveolar air, which may be read on the scale.

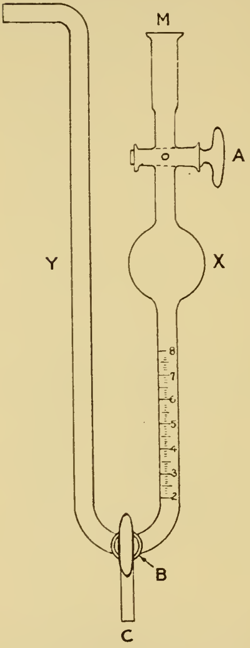

Fig. 132.—Fridericia apparatus.

Procedure. It will be convenient to describe the three positions in which stop-cock *B* may be placed.

Position I. *X* and *Y* communicate, the outlet *C* being closed.

Position II. *Y* and *C* communicate, and *X* is cut off.

Position III. *X* and *C* communicate while *Y* is cut off.

The patient is instructed to breathe into the apparatus through the mouthpiece of arm *X*, the cock *A* being open and *B* being in position I, so that *X* and *Y* communicate while *C* is shut off. At the end of a normal inspiration and without unusual pause, he is directed to blow one hard breath quickly through the apparatus. Cock *A* is closed while *B* is left unchanged. By this process all the atmospheric air is displaced from the apparatus, which is now filled with alveolar air. The apparatus is placed in an upright position in the pail of water at room temperature, leaving the openings of both *Y* and *X* out of the water. On cooling, some of the alveolar air from *Y* is drawn into *X* but there is no danger of the atmospheric air drawn into the upper part of *Y* reaching *X*. In five minutes the stop-cock *C* is turned to Position II, so that *X* is shut off but *Y* communicates with *C*. A rubber bulb is attached to the top of *Y* and about 2 or 3 cc. of the alkali are drawn into the arm *Y* through the opening *C*. The cock *B* is now turned back to Position I so that *X* and Y communicate. The soda is forced into *X* with slight pressure from the bulb. *A*, of course, should be closed during this procedure. While performing this step, the arm *Y* is depressed a little and the arm *X* tipped up so that none of the alveolar air can escape. After this the cock *B* is turned back to Position II so that the soda in arm *Y* may run out through the outlet *C* and so that *X* will be closed completely. The apparatus is inverted and shaken gently for a half-minute or so to expedite the absorption of the CO_2 by the soda, when it is returned to the water-bath and the stopcock *B* is turned under water so that *X* and *C* communicate (Position III), so that the water in the bath may run into the arm *X*. The reading is taken at the end of five minutes. The apparatus is raised so that the water in *X* and that in the bath are at the same level and the level of the water meniscus in *X* is read off on the scale.

No correction need be made for absorbed water vapor since this is small and falls within the limits of error of the method. To transpose the reading to millimeters of mercury under corresponding conditions of temperature and water vapor, it is only necessary to multiply the percentage found by the barometric pressure expressed in millimeters of mercury. When a reading has been made, the apparatus should be cleaned by rinsing with a little dilute acetic acid.

Normal Reading.—The normal reading is a percentage of 5.5, which at sea level corresponds to 42 mm. of mercury.

Marriott's Method.—This has the advantage of being more convenient for bedside determinations, and provides a means for the collection of the specimen from infants and comatose adults.

Principle. When the air, charged with CO_2, is passed through a solution of sodium carbonate until the solution is saturated, the solution contains sodium bicarbonate and dissolved CO_2, the final reaction depending upon the relative amounts of sodium bicarbonate and acid CO_2. This depends entirely upon the tension of the CO_2 in the air which is blown in, and when saturation has been attained is quite independent of the amount of air. The reaction of the resulting solution is changed to the acid side by high tensions of CO_2, to the alkaline side by low tensions, so that the reaction may be used as a measure of the CO_2 tension. In the test, phenolsulphonephthalein is used as the indicator and the standards for comparison are made by mixing definite proportions of acid and alkaline phosphate solutions.

Apparatus and Reagents Required.—(1) Rubber bag of approximately 1500 cc. capacity connected by a short rubber tube to a glass mouthpiece, and a pinch-cock; (2) atomizer bulb; (3) glass tube or pipette drawn out to a fine point; (4) box for color comparison, similar to that used in the Sahli hemoglobinometer but provided with three holes instead of two, with a background of ground glass or opal glass; (5) eight test-tubes containing standard phosphate solutions; (6) standard bicarbonate solution; (7) test-tube of same diameter as the tubes containing the standard solutions, but 100 to 150 mm. long; (8) mask for infants or for comatose patients. This may be adapted from the mask used for ether anesthesia, or may be improvised according to the suggestion of Howland and Marriott. They suggest the use of the rubber mouth-piece of a Hygeia nursing bottle. A piece (about 8 by 10 inches) of thin rubber tissue such as that used by dentists for a dam is perforated in the center with a piece of hot metal. The hole which is made in this manner is stretched and pulled down over the nipple as near the edge as possible and is cemented with rubber cement, reinforcing the union by applying a strip of adhesive plaster about three-eighths or half an inch wide around the edge of the nipple to overlap the rubber tissue. The tip of the nipple is cut off and a short glass tube, about three-eights inch in diameter, is tied into the resulting hole.

Preparation of Standard Phosphate Solutions.—Two solutions are necessary, one a fifteenth-molecular solution of acid potassium phosphate and the other a fifteenth-molecular solution of alkaline sodium phosphate. These are made in the same manner and in the same proportions as the solutions for determining the alkali reserve of the blood. (See page 208.) To them, phenolsulphonephthalein is added as an indicator as there directed. The solutions are mixed in the proportions which are indicated in the table below. The horizontal column bearing the legend "mm." gives the value of the particular solution in terms of carbon dioxide tension when expressed in millimeters of mercury (see Fridericia method).

PROPORTIONS IN WHICH PHOSPHATE SOLUTIONS ARE MIXED.

Mm.	10	15	20	25	30	35	40	45
Acid potassium phosphate, cc.	17.8	25.2	31.0	35.7	40.5	45.0	47.0	50.2
Alkaline sodium phosphate, cc.	82.2	74.8	69.0	64.3	59.5	55.0	53.0	49.8

The solutions may be kept unaltered for some time. They should be preserved, however, in glass which does not give off alkali readily, such as Pyrex, Non-sol, or Jena. Comparison tubes should be of the same sort of glass. To prevent the growth of moulds, a little thymol or tuluol may be added to each solution.

When the solutions have been mixed, a small quantity of each should be placed in a small tube, about 10 by 75 mm. The tubes should be properly labelled, sealed, and kept in a dark place.

The standard bicarbonate solution may be made by dissolving 0.53 gm. of desiccated sodium carbonate in a little water. To this is added 200 cc. of 0.01 per cent. aqueous solution of phenolsulphonephthalein and then enough distilled water to bring the total volume to one liter. If desired, 100 cc. of decinormal sodium hydrate may be placed in a liter flask, 200 cc. of 0.01 per cent. phenolsulphonephthalein added and then water to bring the total volume to the liter mark. This latter solution may be used as it is, since when the carbon dioxide from the air to be tested is blown through, the alkali will be converted to carbonate.

Collection of Alveolar Air.—With an adult who is capable of coöperating to some extent, Marriott recommends the method of Plesch, modified by Higgins. About 600 cc. of air are blown into the rubber bag with the atomizer bulb. The rubber tube is then closed with the clip. The patient, who should be at rest, is directed to breathe easily and naturally and to avoid taking a deep inspiration just before the collection of the specimen is started. At the end of a normal expiration the mouth-piece is placed in his mouth and his nostrils are closed by the operator. He is allowed to breathe back and forth into the bag four times, drawing the air from the bag into his lungs with each inspiration. The operator should indicate the time for inspiration and expiration. At the end of twenty seconds, the tube leading from the mouth-piece to the bag is clamped. The analysis should be made within three minutes since there is a considerable loss of carbon dioxide through the rubber.

When alveolar air is to be collected from children or from unconscious adults, the special mask should be employed. This should be attached to the bag with the tubing. With comatose adults, the bag should be filled with about 1000 cc. of air and the period of breathing lengthened to thirty seconds, since the bag cannot be emptied with each inspiration.

With infants under one year, the amount of air in the bag should be from 250 to 400 cc., enough so that the bag is never emptied completely with the inspiratory effort. The infant should have been quiet for at least one minute previous to beginning the collection of the sample, since vigorous crying leads to a lowering of the tension. While the infant usually cries during the collection of the specimen, this serves to facilitate the mixing of the gases.

After the bag has been filled with the proper amount of air and has been closed with the pinch-cock, the mask is placed over the face, as nearly as possible at the close of expiration. The rubber tissue should be fitted closely about the face to prevent the loss of air, the clamp is opened and respirations are permitted to go on from twenty-eight to thirty-two seconds, when the tube is clipped and the mask removed.

Method of Making Determination.—Into a test-tube of the same diameter as those containing the standard solution but about 100 to 150 mm. long is placed about 3 cc. of the standard carbonate or sodium hydrate solution, containing phenolsulphonephthalein. The piece of glass tubing which has been drawn to a fine point is inserted into the rubber tube attached to the bag. The capillary end of this piece of tubing is then placed in the solution and the clip is released so that the air may bubble through the carbonate solution until there is no further color change. The tube is then stoppered and compared immediately with the standard tubes. If the tube does not match any of the standard tubes exactly, it is placed between the tubes which it most nearly matches and the result is read by interpolation. The readings are in terms of millimeters of mercury. The solutions are prepared to give readings at a temperature between 20 and 25° C. When the room temperature is much higher or lower, the tubes should be placed in water at 25° C. while the air is being bubbled through, when they are removed for color matching.

Findings.—The CO_2 tension in normal adults at rest ranges from 40 to 45 mm. According to Marriott, tensions between 30 and 35 mm. are indicative of a mild degree of acidosis, while a tension below 20 mm. is regarded as a sign of imminent danger. The tension of infants runs from 3 to 5 mm. lower than that of adults.

The CO_2 tension may be affected by conditions other than acidosis. Whenever the respiratory center is stimulated, as by caffein, there is increased pulmonary ventilation and the tension is lowered; while when the center is depressed, the tension is increased.

CHAPTER VII.

BACTERIOLOGICAL METHODS.

General Considerations.—Bacteriological examination is undertaken to determine whether or not microörganisms be present, their nature, and number. The methods include the microscopic study of material obtained from the body (exudates, etc.), the use of cultures, and animal inoculation. The technic for animal inoculation has been given elsewhere.

Direct Microscopic Examination.—In some instances, microscopic examination alone serves clinical purposes (*e. g.*, detection of tubercle bacilli in sputum). The material is spread upon a slide to form a thin film, which is dried, fixed, and stained. Suitable stains have been indicated in the foregoing portions of the text describing the examination of various exudates. General staining methods will be summarized in a following paragraph.

A "hanging drop" preparation may be made for determination of motility. A slide with a circular concavity is usually employed. The surface of the slide around the hollow is smeared with a little vaseline or cedar oil. A drop of the culture is placed in the center of a cover-slip with a platinum loop. The cover-slip is turned over quickly so that the drop hangs from the lower surface, and placed over the hollow in the slide. When properly mounted, the drop hangs in a sealed chamber. If preferred, the drop may be mounted on a slide, which is inverted in a moist chamber.

Cultural Methods.—*Collection of Material.*—Care must be taken to obtain the material free from outside contamination. It should be secured in a sterile receptacle and transferred to appropriate culture media with sterile instruments. Pus is usually secured on sterile cotton swabs. The swab is replaced in the sterile tube which serves as a container. For inoculation, the swab is wiped over the surface of solid medium or dipped into liquid medium.

When few bacteria are present, as in chest fluid, larger quantities of material should be obtained in sterile vessels (bottle, flask, or test-tube), and transferred to appropriate media with a pipette. The technic for blood-cultures has been described elsewhere. Cultures are valueless unless the material has been collected in sterile containers.

Inoculation of Media.—Inoculation may be made into flasks or tubes of liquid media, or into tubes or plates of solid media. When few bacteria are present in thin, fluid material, a considerable quantity should be transferred to the media with a pipette. Pus may be inoculated into liquid media with the cotton swab or platinum loop (Fig. 133, *b*). The inoculating loop must be heated and cooled before and after it comes in contact with infectious material. A sterile cotton swab or platinum spreader is more convenient for making smears on plates, since it is less likely to cut the surface of the medium.

Incubation.—Inoculated media should be placed in an incubator the temperature of which is maintained at constant point by heat-regulating mechanism. The optimum temperature for most pathogenic organisms is body temperature (37.5° C.). Gelatin, however, must be kept at lower temperature, usually that of the room. When electricity is available, an electrically heated and regulated incubator is preferable to gas on account of the freedom from fire hazard and the constancy of temperature.

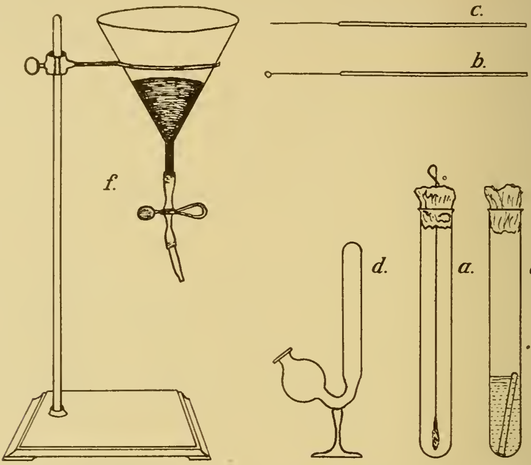

Fig. 133.—Bacteriological apparatus. *a*, swab tube; *b*, inoculating loop; *c*, inoculating needle; *d*, fermentation tube; *e*, Durham fermentation tube; *f*, arrangement for tubing medium.

Study of Cultures.—The cultures are examined after incubation for suitable length of time, which varies with different organisms, but is ordinarily from twenty-four to forty-eight hours. The following methods are at our disposal: (1) Examination of cultures by naked eye; (2) examination of individual colonies by naked eye and with hand lens; (3) Chemical changes in culture medium; (4) morphology and staining reactions of organisms; (5) motility; (6) requirement of anaërobic conditions for growth.

In practical work it is well to examine the culture microscopically first to determine if it be pure or mixed. If pure, the study of the cultures may be completed. If morphological examination of a stained film, however, shows more than one form of organism, the varieties must be isolated.

In the study of cultures, the *gross appearance* of *liquid media* should be noted, including turbidity, pellicle, sediment, gas formation, and odor. *Colonies on solid media* should be described in detail, observing the size, conformation of borders, color, transparency, texture, elevation, and moisture.

Microscopic examination is important and should include at least a study of films stained with Loeffler's methylene blue solution and with Gram's stain. Special methods should be employed for capsules, polar and other bodies, spores, and flagella when indicated. *Motility* should be determined by the examination of a "hanging drop." Among the chemical changes which may be seen and which are helpful in differentiation are, coagulation of milk, change in reaction of media, formation of gas and change in reaction in sugar media by breaking down of sugar, and production of indol.

Isolation of Pure Cultures.—For isolation of varieties, solid media must be employed, so that separate colonies are obtained, from a number of which inoculations may be made into media to obtain pure cultures. The purpose is to secure dilution and distribution of material on solid media so that the colonies will be discrete. *Streak plates* may be made by drawing the inoculating wire or platinum spreader over the surface of a number of tubes of solid media, or in a series of streaks over one or more plates. In this way the material is thinned. A *second method* employs "poured" plates. Agar tubes are melted by placing them in boiling water. The temperature of the water is reduced to about 40° C. when the first tube of the series is inoculated with a loopful of the infectious material. The contents of the inoculated tube are then thoroughly mixed, and two or three loopsful are placed in the second tube. After mixing thoroughly, two or three loopsful from the second are carried into the third tube and so on. The mouths of the tubes are then passed through the flame in turn and the contents poured into sterile Petri dishes, lifting the covers just enough to permit pouring the contents of the tube into the dish. After the medium has solidified, the plates are inverted and placed in the incubator. A *third method* is to take a series of agar tubes and inoculate a loopful of infectious material into the water of condensation of the first tube. A loopful of this is then transferred to the water of condensation of the second tube. After mixing, a loopful is carried to the third tube and placed in the water of condensation. Then all the tubes are tipped so that the water of condensation runs over the surface of the agar and are placed in the incubator. For isolation, the colonies which grow on plates should be viewed with the lowest power of the microscope, placing the Petri dish with cover removed on the stage. Colonies are removed ("fished") with a sterilized platinum needle (Fig. 133, *c*) (not loop), keeping the needle point and the colony in the focus of the lens, so that the point may be seen to touch the colony. The needle is withdrawn without again touching the colony or other object and the material on it is placed in a tube of medium.

Staining Methods.—*A thin film is made* on a slide or cover-slip. Pus may be spread by the "impression method," by smearing the cotton swab over the surface of the slide, or by putting a drop of pus at one end of a slide and spreading it with a second slide (see method for making blood films with slides, page 45). In studying cultures in nutrient bouillon or other liquid media, a drop is placed on a slide or coverslip with a platinum loop (heated in the flame to incandescence and cooled before and after use). When solid media are employed, a drop of normal saline should be put on the slide and a small particle of the growth, picked up with an inoculating needle mixed with it. *The slide is dried in the air*, or by holding it high over (not in) the flame. The danger of overheating will be obviated if the slide be held in the fingers during this step. *Fixation* is accomplished by passing the slide through the flame a number of times with the film side uppermost. The film should not be allowed to scorch. When a brown color is seen, the preparation should be discarded. Instead of heat, the slide may be fixed by covering it with absolute methyl alcohol for two minutes. After fixing, the *stain is poured on* and allowed to remain the necessary length of time, usually one-half to one minute. Except in staining with carbol-fuchsin for the tubercle bacillus, heating is generally unnecessary. The stain is poured off, the slide *washed with* water, and *dried* either by blotting with blotting paper, or preferably by shaking the water off with a motion similar to that employed in shaking down a clinical thermometer, evaporating the residue with moderate heat. When a cover-slip has been employed, it is mounted by placing it over a drop of Canada balsam on a slide, film side down. The cover-slip should be pressed down gently in order to spread out the balsam in a thin layer. When slides are employed, a cover-glass is not necessary.

PREPARATION OF MEDIA.

Tubed media may be secured through retail drug houses, though the price is prohibitive when much work is done. A number of manufacturers offer media in dry powdered form which may be prepared for use by adding water and sterilizing. This is a convenient method for those who do not wish to carry out all steps of preparation and the cost is low. The greatest degree of satisfaction, however, is obtained by the workers when he has media prepared, titrated, and sterilized under his own directions.

After the ingredients have been mixed according to formula, the reaction must be adjusted and the medium cleared, filtered, sterilized and tubed. Directions for these steps are given, followed by formulæ.

Preparation of Glassware.—New glassware may be immersed in a dilute solution of nitric acid, rinsed in tap-water, then in distilled water, and allowed to dry. Glassware containing culture medium or infectious material should be boiled in a 1 or 2 per cent. solution of sodium carbonate and brushed with a test-tube brush, protecting the hands from the strong alkali with rubber gloves. The glassware is then rinsed with tap-water, next with very dilute acid, and finally with distilled water, and allowed to dry. If foreign substances cannot be removed by this method, which is usually sufficient, the glassware may be immersed in bichromate cleaning fluid:

Potassium bichromate	100 gm.
Sulphuric acid, concentrated	120 cc.
Water	1000 "

This solution may be used over and over again.

Flasks, bottles, and tubes should be stoppered with raw cotton rolled to form plugs which will fit the mouths and which will project far enough to be grasped readily by the fingers. (Fig. 133, *e*.)

Types of Apparatus.—Media may be tubed in ordinary *test-tubes,* with or without lip. Tubes of good glass are desirable, in order to prevent the liberation of alkali by repeated heating. *Large square bottles* may be used for making dilutions and as substitutes for more expensive flasks. *Blake bottles* are convenient, since when laid on the side, a large surface of broth or agar is afforded. *Petri dishes* are round, flat, glass plates provided with loosely fitting glass or porous pottery covers. The lower portions serve to hold solidified media. The ordinary size is 10 cm. in diameter. They are used for the isolation of colonies. *Ordinary milk bottles* are convenient for storing media. *Fermentation tubes* with bulb and closed arm may be secured with or without graduation (Fig. 133, *d*). They are expensive and somewhat inconvenient to handle. For most purposes, *Durham fermentation tubes* may be substituted (Fig. 133, *e*). A small test-tube, about 1 by 6 cm. is dropped, open end down, into a culture tube containing 5 to 7 cc. of medium. The fluid completely fills the small tube during sterilization. After the medium is inoculated, any gas formed will rise in the small tube, displacing from it part of the medium.

Swab-tubes may be made by taking pieces of copper wire or wooden applicators the length of which exceeds that of the ordinary test-tube, wrapping one end tightly with absorbent cotton, and placing the swab in a test-tube. The cotton closing the mouth of the test-tube is wrapped about the upper end of the wire (Fig. 133, *a*). The outfit may be sterilized by dry heat.

Adjustment of Reaction.—The reaction of media is of great importance since bacterial growth is susceptible to variations in acidity or alkalinity. Two methods for adjusting the reaction are given here, the titrimetric method which has been employed for many years, and colorimetric determination of the hydrogen-ion concentration.

Titrimetric Method.—With a volumetric pipette, put 5 cc. of the medium in a white porcelain evaporating dish and add 45 cc. of distilled water. Boil briskly for a minute. Add 1 cc. of phenolphthalein solution (0.5 gm. dissolved in 100 cc. of 50 per cent. alcohol) and titrate at once by adding $\frac{N}{20}$ sodium hydrate solution from a burette (graduated in 0.1 cc.) until a faint but distinct color marks the end-point, stirring the medium constantly with a glass rod while adding the alkali. According to the standard methods of the American Public Health Association this tint may be described as a combination of 25 per cent. red (wave length approximately 658) with 75 per cent. white as shown by the disks of the standard color top made by the Milton Bradley Educational Company of Springfield Massachusetts.

Should the medium be alkaline, that is, give a red color on the addition of the phenolphthalein indicator, titration should be carried out by adding $\frac{N}{20}$ hydrochloric acid until the pink color disappears and the medium becomes colorless.

The amount of acid or alkali required for neutralization is readily calculated. While $\frac{N}{20}$ alkali or acid solution is used for titration, the medium is neutralized with normal solution. It is evident that if X quantity of $\frac{N}{20}$ NaOH be required to neutralize 5 cc. of medium, then X cc. normal NaOH will neutralize 100 cc., and 10 times this amount will neutralize 1 liter.

Reaction is expressed with reference to the phenolphthalein end-point (Fuller scale). Alkaline media are recorded with the minus sign (−) before the percentage of normal acid solution which would be required for neutralization, while acid media are recorded with the plus sign (+) before the amount of normal alkali which would be required for neutralization. The actual adjustment of reaction is determined by the use to which the medium is put. For examination of water, the standard methods of the A. P. H. A. specify that the reaction shall fall between +0.5 and +1.0. When titration shows that the reaction is within this range, no adjustment is necessary. For general routine work, slight alkalinity, neutrality or an acidity not exceeding +1.0 is customary.

Example.—Suppose the desired reaction be +1.0. Titration shows that 2.5 $\frac{N}{20}$ NaOH is require to neutralize 5 cc. Then 2.5 cc. of normal NaOH is required to neutralize 100 cc. Therefore, the reaction of the medium before adjustment is + 2.5 (since this is the percentage of normal alkali required for neutralization). A reaction of + 1.0 is desired, however, so that to each 100 cc. of media, 1.5 cc. ($2.5 - 1.0 = 1.5$) of normal NaOH should be added

Colorimetric Determination of Hydrogen-ion Concentration.—The titrimetric method is open to a number of objections. One is that the end-point is not sharp due to the action of buffer-substances (proteins, amino-acids, and certain salts) which take up the hydrogen ions so that the addition of moderate quantities of acids make a comparatively small change in the reaction. Another is that a great difference is made by personal equation of the operator on account of variations in sharpness of color-sense. The true reaction depends upon the amount of hydrogen or hydroxyl ions which are present. Hydrogen-ion concentration is best determined by the hydrogen electrode, but such apparatus is not available except in well equipped laboratories. In clinical laboratory practice, sufficiently accurate results can be obtained by colorimetric methods. (Brief consideration has been given to hydrogen-ion concentration in the chapter on Blood, page 208).

The hydrogen-ion concentration is expressed in terms of a negative logarithm as the "hydrogen exponent," and is usually written pH. With such minus logarithms, two facts should be remembered. The actual amount of hydrogen ions decreases as the pH increases. In other words, $pH=8.0$ represents a less acid solution than $pH=7.0$. Furthermore, the amounts are logarithms, and not simple quantities, so that $pH=3.0$ is a solution containing 10 times as many hydrogen ions as $pH=4.0$.

The hydrogen exponent of water is $pH=7.0$, while that of blood fluids is in the region of $pH=7.5$. It is usually desired to prepare media with reaction in the neighborhood of $pH=7.0$ to $pH=7.5$. While various indicators are required at different ranges, for example, thymol blue between $pH=1.2$ and $pH=2.8$, and bromphenol blue between $pH=2.8$ and $pH=4.6$, phenolsulphonephthalein (phenol red) is the indicator whose color changes are best appreciated within the range from $pH=7.2$ and $pH=8.0$. The initial reaction of a medium may be determined by preparing solutions of known hydrogen-ion concentration, adding to them small amounts of the indicator. The tint produced serves as a standard to which the medium is brought by titration. A precisely similar quantity of indicator is added to a measured quantity of the medium to be tested and acid or alkali is run in from a burette until the colors of the solution and of the standard match. In matching the colors, the brown tint of the medium is compensated for by interposing tubes of untreated medium between the standards and the light.

Procedure for Determination of Hydrogen-ion Concentration.—A colorimetric method was utilized by Hurwitz, Meyer, and Ostenberg. This is given here, except that titration is performed with 5 cc. samples rather than 3 cc. samples. *The standard colors* may be prepared according to the directions of Levy, Rowntree and Marriott after Sörensen. Two standard phosphate mixtures are necessary. $\frac{1}{15}$ *molecular acid or primary potassium phosphate;* 9.078 gm. of pure recrystallized salt (KH_2PO_4) is dissolved in freahly distilled water (freed from ammonia by boiling) and made up to 1000 cc.

$\frac{1}{15}$ *molecular alkaline or secondary sodium phosphate.*—The pure recrystallized salt ($Na_2PO_4.2H_2O$) is exposed to the air for about two weeks, protected from the dust. Ten molecules of water are given off so that the formula of the salt obtained is $NaHPO_4.2H_2O$. Of this, 11.876 gm. are dissolved in distilled water and the volume is made up to one liter with water. If the salt be sufficiently pure, the solution should give a deep red with phenolphthalein.

The solutions are mixed in the proportions shown in the following table to obtain the indicated pH:

pH	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	8.0	8.2	8.4
Primary potassium phosphate sol., cc.	73	63	51	37	32	27	23	19	15.8	13.2	11.0	8.8	5.6	3.2	2.0
Secondary sodium phosphate sol., cc.	27	37	49	63	68	73	77	81	84.2	86.8	89.0	91.2	94.4	96.8	98.0

Five cc. of each solution are placed in Pyrex glass test-tubes of similar size, 0.5 cc. of 0.01 per cent. aqueous solution of phenolsulphonephthalein[1] are added to each tube, and the ends of the tubes sealed.

Color comparison is made in a comparator, or wooden box with a double row of holes, three in a row, so arranged that the tubes are viewed with transmitted light. The comparator shown (Fig. 134) is a modification by Cole and Onslow of one suggested by Hurwitz, Meyer, and Ostenberg, and by Walpole.

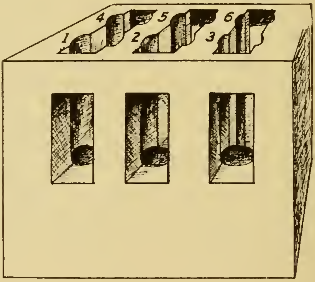

Fig. 134.—Cole and Onslow's color comparator.

In addition, the following apparatus and reagents are required: (1) Microburette, improvised from a calibrated 1.0 or 2.0 cc. pipette, divided into 0.01 cc. A fine glass capillary tip should be attached to the lower end with rubber tubing and the delivery of liquid should be controlled with a pinch-cock; (2) bacteriological pipette to deliver 0.5 cc. and a volumetric pipette to deliver 5.0 cc.; (3) clean Pyrex glass test-tubes of same size as those used for standards; (4) phenol-red solution, 0.01 per cent. This should be the same solution as that used in making standards. (5) $\frac{N}{10}$ NaOH, made from the normal solution which is finally added to the media for adjustment of reaction. To prepare, place 50 cc. of $\frac{N}{1}$ solution in a 1000 cc. volumetric flask and add distilled water to the 1000 cc. mark. Both alkali solutions should be kept in bottle whose inner surfaces have been coated with paraffin.

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[1] This may be prepared by adding 59 cc. of distilled water to 1 cc. (= 6 mg.) of the standardized solution prepared by Hynson, Westcott and Dunning, of Baltimore, Md., for testing kidney function. This solution should be used also as the indicator in carrying out the titration.

Titration.—Measure 5 cc. of the medium into one of the test-tubes and add 0.5 cc. of 0.01 per cent. phenol-red solution. Place this in the middle hole (No. 2) of the front row in the comparator (Fig. 134). In the hole directly in back (No. 5) place a tube with distilled water. If a reaction of pH = 7.5 be desired, place in the hole on the left (No. 1) standard tube 7.4 and in the hole on the right. (No. 3) standard tube 7.6. In the two holes back of these (Nos. 4 and 6), place tubes containing uncolored medium. If the medium be more acid than is desired, add $\frac{N}{20}$ NaOH from the microburette until the tint is between that of the two standard tubes, viewing the tubes with transmitted light. Shake the tube to mix contents thoroughly. When the smount of alkali required to bring 5 cc. of the medium to the desired tint is known, it is easy to calculate the amount of $\frac{N}{1}$ NaOH required to bring the bulk of the medium to the required hydrogen-ion concentration. If, for example, 0.3 cc. of $\frac{N}{20}$ NaOH were required to bring the desired color with 5 cc., 3.0 cc. of normal NaOH would be needed for 1 liter.[1]

Media become more acid after sterilization. This is particularly marked in media containing sugar. It is suggested that sugar solutions be sterilized separately and added to the medium after the nitrogenous part of the medium. Solid media may be liquified by heat before making the colorimetric determination. After alkali has been added to the medium, the reaction should be determined again as a control on the accuracy of the work.

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[1] It is stated by Hurwitz, Meyer and Ostenberg that the amount of normal alkali to be added cannot be calculated accurately in this way because of certain variables, *i. e.*, alteration in volume because of the addition of concentrated rather than dilute alkali, the difference in dilution of the "buffers" by alkali solutions of different strengths, and the possible lack of correspondence between $\frac{N}{1}$ and $\frac{N}{20}$ solutions. They recommend plotting a curve from the data obtained by the titration of average laboratory samples. The curve is constructed in the following manner: Add to 25 cc. portions from each of the two media, one originally acid and one alkaline in reaction, graduated amounts of $\frac{N}{20}$ alkali and acid respectively in amounts of 0.1 cc. to the first portion, 0.2 cc. to the second, and so on up to 0.5 cc. to the last. From each of these portions, take 3 cc. and add 0.3 cc. of the phenol-red indicator solution. These serve as standards. Take another 3 cc. sample of the medium to which neither acid nor alkali has been added and add 0.3 cc. of indicator solution, titrating with $\frac{N}{20}$ alkali or acid until the color matches successively that of each of the five tubes containing normal alkali or acid. Plot the amounts of $\frac{N}{20}$ solution required per 3 cc. as abscissæ and the corresponding amounts of $\frac{N}{1}$ solution *per liter* as ordinates. With the curve which was plotted by Hurwitz *et al.*, if 0.46 cc. of $\frac{N}{20}$ alkali were used in titration to secure the desired pH, then 9 cc. of $\frac{N}{1}$ alkali solution should be added to a liter of medium. After the curve has been prepared for a given set of solutions, titration of a 3 cc. portion may be carried and the amount of $\frac{N}{1}$ solution necessary to adjust the reaction of one liter may be read directly from the curve. (See Fig. 135.)

Clearing Media.—This is accomplished by the coagulation of dissolved proteins by heat, the coagulum enmeshing fine particles. When medium is devoid of protein, white of eggs must be added. The white of one or two eggs is beaten with about 20 cc. of water and stirred into

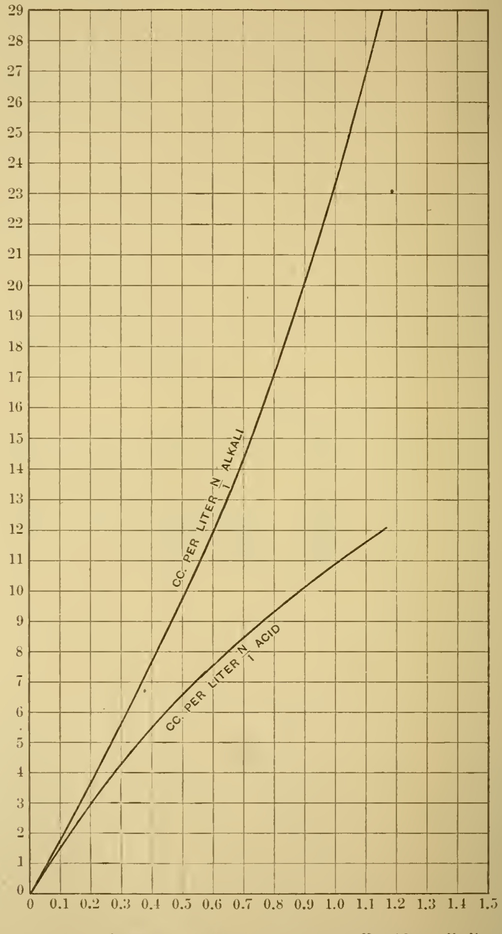

Fig. 135.—Curves indicating the actual amounts of $\frac{N}{1}$ acid or alkali necessary to adjust 1 liter of medium to the desired hydrogen-ion concentration, as indicated by the amount of $\frac{N}{20}$ acid or alkali used. (Johns Hopkins Hospital Bulletin.)

the medium, which should be cooled to about 55° C. It is then heated in the Arnold sterilizer for forty-five minutes, when it is taken out, thoroughly shaken to break up the coagulum, and again heated in the Arnold sterilizer for fifteen minutes. This is referred to in the following directions as "clearing with egg." After coagulation, the medium is filtered.

Filtering.—Fluid media may be cleared by filtration through close filter-paper. It is advisable to plait the paper or to use a corrugated funnel. Should coagulum be present, cotton should be employed. A spiral of wire is placed in the bottom of a large glass funnel and across it are laid at right angles two long thin strips of absorbent cotton. The ends are made to adhere to the sides of the funnel by moistening them with water. The medium is run in along a glass rod to direct the stream so that it will not break through the filter. If it be cloudy as it first comes through it should be poured back until the pores are filled with coagulum. The funnel should be heated before use to prevent solidification. A hot-water jacket, fitting around the funnel, is most convenient.

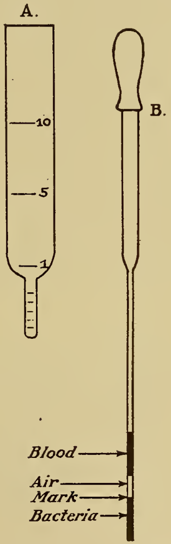

FIG. 136.—*A*, Hopkin's tube; *B*, Wright's pipette.

Tubing.—After medium has been cleared and filtered, it is liquified by heat and poured into a glass funnel. Rubber tubing should be attached to the end of the funnel and a pinch-cock used to control discharge (Fig. 133, *f*). The medium is run into tubes, placing about 5 to 6 cc. in each tube. The tubes are stoppered and sterilized.

Sterilization.—*Hot Air.*—All glassware and metal instruments may be sterilized in an oven or hot air sterilizer, preferably one of the Lautenschlager type for one hour at 150° C. Flasks, bottles, and tubes should be plugged with raw cotton. Petri dishes may be placed in a cylindrical copper box or may be wrapped with paper in sets of two or three. Pipettes may be wrapped in paper. One exposure to this temperature kills both spores and bacteria.

Steam.—Steam is usually used for sterilization of media. In the Arnold sterilizer, the steam rises to the top and condenses, the water falling down the wall to the vessel below where it is boiled. Two types are available, one in which a cover is lifted off and the other, with a door on the side, Boston Board of Health type. Sterilization with this apparatus is conducted on the fractional plan, that is, at a temperature of 100° C. for twenty minutes on each of three successive days. This is necessary since a single sterilization at 100° C. kills the bacteria but not the spores. The spores develop during the time between sterilizations and are destroyed by the subsequent heating. Media containing sugar are usually sterilized in the Arnold rather than in the autoclave to avoid the possibility of breaking down sugar. Although an autoclave is expensive and not absolutely necessary, it is very convenient. The higher temperatures which can be attained kill all spores so that it is possible to complete the sterilization with one exposure.

Media in test-tubes are subjected usually to 15 pounds pressure (121.3° C.) for fifteen minutes. Flasks containing larger quantities of fluid should be left in for a longer period of time, from thirty to sixty minutes, depending on the amount of fluid to be sterilized. The temperature varies with the pressure, the equivalents being shown in the following table, taken from Hiss and Zinsser:

1 pound of pressure equivalent to temperature of 102.3° C.
5 pounds of pressure equivalent to temperature of 108.8° C.
10 pounds of pressure equivalent to temperature of 115.°6 C.
15 pounds of pressure equivalent to temperature of 121.3° C.
20 pounds of pressure equivalent to temperature of 126.2° C.
30 pounds of pressure equivalent to temperature of 134.6° C.

Cotton, gauze, and glass hypodermic instruments may be sterilized either by hot air or steam, although the latter is preferable since it obviates the possibility of damage due to excessive heat.

Slanting Solid Media.—Solid medium is usually slanted after sterilization. As soon as the tubes are removed from the sterilizer, they are laid in a nearly horizontal position, the upper ends being raised slightly by supporting them on a length of glass tubing or a flat strip of wood. The medium should not be allowed to come in contact with the stoppers. Agar should be left in this position for at least twenty-four hours.

Storing Media.—Media should be kept in the refrigerator. When only small quantities are used at a time, the bulk may be kept in bottles or flasks, the contents of which may be melted, tubed and resterilized in small lots as needed. Evaporation may be prevented by covering the cotton plugs with rubber caps or tin-foil, or by pushing in the plugs and by stoppering the tubes with corks or melted paraffin.

FORMULÆ FOR MEDIA.

The Standard Methods of the American Public Health Association gives the following requirements for the materials used in making media. Liebig's meat extract and either Armour's, the Digestive Ferments Company's, or Fairchild's peptone should be used. Distilled water should be employed. The sugars should be of the highest purity obtainable. The agar must be of the best quality and should be dried one-half hour at 105° C. before weighing. Since many brands contain a large quantity of sea salts, it is well to soak the agar in water and drain before use. Gelatin should be of light color, containing only a trace of arsenic, copper, and sulphides, and of such melting-point that a 10 per cent. solution will melt at 25° C. or over. It must be dried for one-half hour at 105° C. before weighing. Reagent litmus of the highest purity or azolitmin should be used, not litmus cubes. All chemicals should be of the highest purity.

Meat Extract Broth (bouillon.).

Meat extract	5 gm.
Peptone	10 "
Sodium chloride	5 "
Water	1000 cc.

Put ingredients in an agate or enamelled pan, and weigh vessel. Heat over a flame until dissolved, stirring constantly. Make up lost weight by adding water. Titrate and adjust to proper reaction. Heat over free flame for five minutes. Sterilize and filter. If medium does not clarify completely, clear with egg.

Meat Infusion Broth (bouillon.).

Fresh meat infusion.

Lean beef or veal	500 gm.
Water	1000 cc.

Secure about one and a quarter pounds of meat, free from fat and tendinous material and force through the meat grinder twice. Place meat and water in an agate pan in the ice-chest over night. Strain through cheese-cloth. Add water to restore volume to 1000 cc. Use this in making the broth.

Meat infusion broth.

Fresh meat infusion	1000 cc.
Sodium chloride	5 gm.
Peptone	10 "

Weigh the vessel containing the mixture. Warm over a flame to dissolve peptone, but do not allow the temperature to rise above 50° C.

Restore lost weight by adding water. Determine reaction and adjust to neutral. Heat in Arnold sterilizer for thirty minutes. Shake well and heat again for fifteen minutes. Weigh and restore lost weight with water. Titrate and adjust to desired reaction. Heat if acid or alkali be added. Filter through cotton, tube, and sterilize.

Sugar-free Broth.—Prepare a meat-infusion broth up to the point following heating in the Arnold sterilizer. Do not adjust the reaction but filter through cheese-cloth and bring up to original volume with water. Cool to room temperature and to each liter of medium add a tube of twenty-four-hour growth of B. coli communis. Stopper flask with cotton and place in the incubator for twenty-four hours. Sterilize in the autoclave at 15 to 18 pounds pressure for one hour. Restore volume with water. Adjust reaction to neutral. Heat in Arnold sterilizer for thirty minutes, filter through a double layer of filter paper, tube and sterilize.

Carbohydrate Media.—These are usually made with sugar-free broth as a basis. If beef-extract broth be used, however, removal of carbohydrate is unnecessary. Carbohydrates are generally used in 1.0 per cent. concentration, that is, 1.0 gm. is used for each 100 cc. of media, and 1 cc. of saturated aqueous solution of neutral red, is added. The media should be heated in the Arnold sterilizer for twenty minutes on three successive days.

If desired, a 10 per cent. solution may be made of most of the carbohydrates. Sterilize this at 100° C. for one and a half hours, and add 10 cc. with a sterile pipette to each 100 cc. of sterile media. Tube mixture and sterilize at 100° C. for thirty minutes. Incubate tubes at 37° C. for twenty-four hours to test sterility.

Dunham's Peptone Solution.

Sodium chloride	5 mg.
Peptone	10 gm.
Water	1000 cc.

Dissolve by heating and filter. Adjustment of reaction is not required.

Calcium Carbonate Broth.

Calcium carbonate (powdered)	10 gm.
Nutrient broth	1000 cc.

Tube and sterilize.

Litmus Milk.—Steam fresh milk in an Arnold sterilizer for fifteen minutes and place in the ice-box over night. The cream rises to the surface. Siphon off the lower layer of fat-free milk and add sufficient litmus solution to give a deep lavender color. A solution of azolitmin is preferable. Tube, placing about 10 cc. in each tube. Sterilize in the Arnold sterilizer for twenty minutes on three successive days.

Hiss' Inulin Serum-water.—Dissolve 10 gm. inulin in 750 cc. of water and sterilize in the autoclave for fifteen minutes at 15 pounds pressure. Add 250 cc. of clear beef serum and enough aqueous litmus solution to give a deep lavender. Mix thoroughly, tube with about 10 cc. in each tube, and sterilize in the Arnold sterilizer for twenty minutes on three successive days. (For method of obtaining beef-serum, see Loeffler's Blood Serum.)

Meat Extract Gelatin.

Meat extract	5 gm.
Peptone	10 "
Sodium chloride	5 "
Gelatin	120 "
Water	1000 cc.

Weigh mixture with containing vessel and dissolve ingredients by warming. Restore lost weight with water and adjust reaction to desired point. Cool to 60° C. and clear with egg. Heat in Arnold sterilizer for thirty minutes, shake, heat again for fifteen minutes, restore lost weight with water, and filter through cotton. Tube and sterilize in Arnold sterilizer for thirty minutes on three successive days.

Meat-infusion Gelatin.—Make meat-infusion broth as directed, adding 120 gm. of gelatin, mixing all ingredients with water at same time. Carry through other steps as outlined, tube in sterile test-tubes, and sterilize in Arnold sterilizer for thirty minutes on three successive days. Longer sterilization may interfere with solidification of the media.

Meat Extract Agar.

Meat extract	5 gm.
Peptone	10 "
Sodium chloride	5 "
Agar	15 "
Water	1000 cc.

Mix in an agate pan. Weigh vessel with contents. Heat over a free flame until agar is dissolved. This may require thirty to forty-five minutes. Restore weight with water. Determine reaction and adjust to desired point. Cool to 60° C. and add whites of two eggs, thoroughly beaten with a little water. Mix well. Heat in Arnold sterilizer for thirty minutes, shake or stir thoroughly, and heat again for fifteen minutes. Restore lost weight with water. Titrate and again adjust reaction if necessary. Filter through cotton, tube, and sterilize.

Meat-infusion Agar.

Double-strength Meat-infusion.

Lean beef or veal	500 gm.
Water	500 cc.

Secure about one and one quarter pounds of meat free from fat and tendinous material and run through the meat grinder twice. Place the meat and water in an agate pan in the ice-chest over night and strain through cheese-cloth in the morning. Add water to restore the volume to 500 cc. Use this in making broth as indicated below.

Meat-infusion Agar.

Peptone	10 gm.
Sodium chloride	6 "
Double-strength meat-infusion	500 cc.

Add peptone and salt, weight the vessel, warm over free flame to dissolve, keeping the temperature below 50° C. Restore lost volume with water, titrate, and neutralize.

Dissolve 15 gm. agar in 600 cc. water by heating over a free flame for thirty to forty-five minutes. Cool to about 50° C. and add to the meat-infusion broth. Weigh the mixture. Heat in Arnold sterilizer for thirty minutes, shake thoroughly, heat again for fifteen minutes, and adjust to former weight by adding water. Determine volume, titrate, and adjust reaction to desired point. Boil over free flame for two minutes, stirring constantly. Filter through cotton, passing back through same filter until clear. Tube and sterilize.

Glucose Agar.—Prepare agar by one of the preceding methods. Add 10 gm. of glucose to 1000 cc. of filtered clarified agar. Tube and sterilize in the Arnold sterilizer for twenty minutes on three successive days.

Avery's Blood Oleate Agar.—See page 360.

Blood Agar.—See pages 359 and 362.

Agar and Broth for Pneumococcus Culture.—See page 362.

Hydrocele or Ascitic Fluid Agar.—Obtain hydrocele or ascitic fluid under aseptic conditions. Melt agar by placing the tubes in boiling water. Reduce temperature of water to 50° C. and with a sterile pipette add to each tube 1 or 2 cc. of body fluid. Allow tubes to solidify in slanting position.

Thomson's Medium for Gonococcus.—Prepare 2.5 per cent. nutrient agar in the usual way, using 1 per cent. peptone. Adjust reaction to +0.6 acid. Instead of adding sodium chloride only, add salts employed in Ringer's solution, namely, 9 gm. of sodium chloride, 0.25 gm. of calcium chloride, 0.42 gm. of potassium chloride to each liter of medium. Add 2.5 per cent. of glucose. Clear and filter agar, place 4 cc. in tubes, and sterilize. When ready to add the human serum, melt agar, cool to about 50° C., and add 1 cc. of human serum to each tube. Mix the contents of the tube thoroughly by rolling the tube between the palms, and allow to solidify in the slanting position.

Human serum is readily obtained by venipuncture (see page 126). drawing blood into a sterile tube containing a few crystals of sodium citrate. Centrifugalize blood, remove serum with a sterile pipette and add to the agar tubes. Thomson states that taking blood from an individual under mercury or arsenic treatment does not interfere with growth of the gonococcus.

Petroff's Medium.—See page 358.

Loeffler's Blood Serum.—Obtain beef blood from the slaughter-house in a large enamel-ware pail. Care should be taken to keep the blood free from hair and other foreign substances. Separate the clot from the side of the vessel with a glass rod and place the pail in the ice-chest over night. In the morning, remove the serum with a pipette. If it be cloudy, put it in a large centrifuge tubes and throw down the cells.

A 1 per cent. glucose meat-infusion broth is required. The reaction should be made neutral to phenolphthalein. Mix 3 parts of serum with 1 part of dextrose broth, and tube in small tubes. Place tubes in a slanting position in Koch serum-coagulator (inspissator) and heat gradually until the temperature reaches 90° C. Keep temperature at this point until the medium has coagulated completely. It is important that the temperature be raised slowly to prevent formation of bubbles in the media. Sterilize on the second and third days in the Arnold sterilizer, raising temperature slowly to the boiling-point and maintaining it at this point for twenty minutes.

In the absence of an inspissator, the medium may be solidified in the autoclave by raising the temperature very gradually to 105° C. or in the Arnold sterilizer by covering the top with a cloth instead of the usual cover.

Dorset's Egg Medium.—Wash eggs carefully with water and then with 5 per cent. phenol solution. Break them and pour contents into a sterile Erlenmeyer flask. Add 25 cc. of water for every 4 eggs. Mix and strain through sterile cheese-cloth. Tube and inspissate as with Loeffler's blood serum.

Potato.—Scrub large potatoes under running water, and cut cylinders with an apple corer. Remove the skin from both ends and divide the cylinders with an oblique cut to form wedge-shaped pieces. Wash them in running water for several hours and place in tubes with the large end down. Sterilize in Arnold sterilizer for twenty minutes on three successive days.

Endo's Fuchsin Agar.—(Standard Methods, American Public Health Association.)

Beef extract	5 gm.
Peptone	10 "
Agar	30 "
Water	1000 cc.

Place in flask, weigh flask and contents, and boil on water-bath until the agar is dissolved. Make up loss of weight by adding water. Cool mixture to 45° C. in cool water-bath and then warm to 65° C. in same bath without stirring. Make up lost weight, titrate, and if necessary adjust reaction to between neutral and + 1. Filter through cotton until clear. Distribute between flasks in 100 cc. quantities. Sterilize in autoclave for fifteen minutes at 15 pounds pressure. Make a 10 per cent. solution of basic fuchsin with 95 per cent. alcohol. After this has stood for twenty hours, decant the supernatant fluid and keep as a stock solution.

When ready to make plates, melt a flask containing 100 cc. of agar in the Arnold sterilizer or on a water-bath. Dissolve 1 gm. of lactose in 15 cc. of distilled water, with heat if necessary, and 0.25 gm. anhydrous sodium sulphite in 10 cc. water. Add 0.5 cc. of the stock fuchsin solution to this solution of sodium sulphite and then mix the fuchsin-sulphite solution with the lactose solution. Add the resulting mixture to the melted agar. The sulphite solution must be fresh. Plates are poured and allowed to harden in the incubator before use. On this media typhoid colonies appear as clear, colorless dew-drops while typical colon colonies are colored red with fuchsin.

Russell's Double Sugar Agar.—Prepare plain agar, making the concentration of agar 2 to 3 per cent. The reaction should be about +0.7. Add 5 per cent. aqueous solution of litmus to produce a distinct purple color. Then add normal sodium hydrate until the mixture is neutral to litmus. Add dextrose to make a concentration of 0.1 per cent. and lactose to make a concentration of 1 per cent. Before adding dissolve sugar in a small quantity of water. Tube and sterilize in the Arnold sterilizer for twenty minutes on three successive days. Slant the tubes and store in a dark place.

Inoculate the tube by stroking the surface and stabbing into the butt. After incubating from eight to eighteen hours, the typhoid bacillus surface growth is filamentous and colorless on a blue background, while that in the lower part of the butt is a deep red. In the imperfect anaërobic conditions in the butt, the organism obtains its oxygen by breaking down dextrose with the formation of acid. No acid is formed in the presence of oxygen. The colon bacillus shows abundant gas and acid formation, the medium being reddened throughout. The paratyphoids have a surface growth like typhoid while the butt shows reddening and a few small gas bubbles.

Levine's Simplified Eosine-methylene-blue Agar.—

Agar	15 gm.
Peptone	10 "
Dipotassium phosphate	2 "
Water	1000 cc.

Boil the ingredients until dissolved and make up the loss of water due to evaporation. Place measured quantities into bottles or flasks and sterilize in autoclave at 15 pounds pressure for from fifteen to twenty minutes.

Just before using, the agar is melted and to each cubic centimeter is added the following:

Lactose (20 per cent. solution)	5 cc.
Eosine, yellow (2 per cent. aqueous solution)	2 "
Methylene blue (0.5 per cent. aqueous solution)	2 "

It is desirable to have the last three solutions sterilized in the autoclave before adding to medium.

After agar has been mixed, pour into plates and allow to harden. Inoculate by streaking surface. Colonies of typhoid bacilli are white, those of colon are blue.

MacConkey's Bile-salt Agar.

Sodium glycocholate	8 gm.
Peptone	20 "
Lactose	10 "
Agar	15 "
Water, to make	1000 cc.

Dissolve the agar and peptone in the water, filter, and clear. Add the lactose and sodium glycocholate, tube and sterilize in the Arnold sterilizer on three successive days for twenty minutes.

Colonies of B. typhosus produce no change while those of B. coli produce acid and cause precipitation of bile salts.

Krumwiede's Brilliant Green Agar.

Beef extract	3 gm.
Sodium chloride	5 "
Peptone	10 "
Agar	15 "
Water	1000 cc.

Dissolve in autoclave, clear and filter. Add alkali to make slightly alkaline to litmus, bottle in 100 cc. quantities, and sterilize.

When medium is to be used, melt 100 cc. and add 1 cc. of Andrade's indicator. Adjust the reaction to the neutral point to the indicator, that is, until the red color of the hot medium fades completely on cooling. The reaction point is best determined by titration of a small quantity.[1]

Add 5 cc. 20 per cent. sterile lactose solution, 5 cc. of 0.1 per cent. sterile glucose solution, and brilliant green solution, either 0.2 cc. or 0.3 cc. of 0.1 per cent. dilution per 100 cc. of melted agar. The medium is poured into 6 Petri dishes to give thick plates. The plates are left uncovered until dry. Any variation in the composition of the medium requires a readjustment of dye concentration. Brilliant green inhibits the growth of all Gram-positive and many Gram-negative cocci, and shows differential action with colon-typhoid group, colon bacilli being extremely susceptible to the dye, while typhoid bacilli grow as a snow flake colony with delicate serrated edge, which may be slightly tinged with red from acid production.

Conradi's Bile Medium.

Peptone	20 gm.
Glycerol	100 cc.
Ox-bile	900 "

Ox-bile is obtained from the abattoir; glycerol and peptone are added; the mixture is tubed and sterilized in the Arnold sterilizer for twenty minutes on three successive days.

Jackson's Lactose-bile Medium.

Peptone	10 gm.
Lactose	10 "
Ox-bile	1000 cc.

The medium is tubed in fermentation tubes and sterilized in the Arnold sterilizer for twenty minutes each on three successive days.

Robertson's Cooked Meat Medium for Anaërobes.—Mince 8 ounces of bullock's heart very fine and grind in a mortar. Add 8 ounces of water and heat slowly, to cook the meat thoroughly. Add normal sodium hydrate until the mixture is alkaline to litmus. Divide into tubes and autoclave.

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[1] Andrade's indicator is prepared by adding 16 cc. normal sodium hydrate to 0.5 per cent. aqueous solution acid fuchsin. The dye is alkalinized slowly and turns red in the presence of acid.

APPENDIX.

EXAMINATION OF A LARGE NUMBER OF URINE SPECIMENS IN A HOSPITAL LABORATORY.

The standing orders of most well-organized hospitals prescribe a certain routine, such as the submission of first specimens immediately upon admission, of daily and weekly specimens, and of preoperative and postoperative specimens in surgical cases. The examination of preoperative specimens is of especial importance, since the surgeon's choice of the anesthetic is often based upon the report. When twenty-four-hour specimens are desired they should be collected in the wards and placed in large containers, in which the entire specimen may be sent to the laboratory, where the quantity may be measured accurately by the laboratory worker. One of the greatest difficulties in having reliable urinalyses made in hospitals is the extreme difficulty that is encountered in training orderlies and even nurses in the importance of the saving of all the urine.

The specimen should be sent to the laboratory properly labelled. The label should bear the name of the patient, the name of the attending or house physician, a notation as to whether it be a single or a twenty-four-hour specimen, the ward or room number, and the date.

Nurses should be instructed to have urine voided and sent to the laboratory only in thoroughly cleaned vessels, and to send only specimens free from admixture with fecal material.

In most hospitals it is the practice to send the specimens from the different wards to the laboratory in the morning. The person whose duty it is to examine them is confronted with the problem of making a large number of examinations with as great an economy of time as is consistent with careful work. A definite routine is necessary. The specimens should be given working serial numbers, beginning each day with 1. This should be written on the label which accompanies the specimen. A wooden test-tube rack is convenient. This should be made with five rows of holes and the holes in the front row should be numbered serially, beginning with 1. The holes in the front row should be large enough to receive tubes 2.5 by 18 cm. while those in the other four rows need only be large enough to receive test-tubes of the ordinary size. The large tubes in the front row serve to float the hydrometer. Before beginning the tests, one row of tubes should be charged with Benedict's solution (5 cc. in each tube) and one with nitric acid (5 cc.

in each tube). The tubes in one row should be left empty, and the fifth row should be filled with 15 cc. centrifuge tubes, numbered serially to correspond with the numbers of the tubes in the front row.

The specimens may be poured from the containers into the large test-tubes which bear corresponding numbers. These tubes serve as reservoirs and are convenient for taking the specific gravity. Small bits of red and blue litmus paper are dropped into the specimens. The hydrometer is moved from one large tube to the next, rinsing after each reading under running water. A hydrometer made after the principle of the battery tester (see Fig. 61 *b*), is serviceable. As the reaction and specific gravity are taken, they should be noted in the record. Before beginning the chemical examination, it is well to place samples of urine in the numbered centrifuge tubes and to allow centrifugalization to proceed while the chemical work is being carried out. At the close of the chemical procedure, the centrifugalized specimens will be ready for microscopical examination.

For albumin tests, enough of each specimen may be poured into the row of empty tubes to fill the tubes nearly to the top. These portions are used for the test by the heat and acetic acid method. A few cc. of each specimen are run over the nitric acid in the tubes which have been previously charged with this reagent for the cold nitric acid test. Into the row of tubes which have been charged with Benedict's solution there should be added 5 to 8 drops of urine, using a medicine dropper which may be rinsed with water after use with each specimen. If Fehling's solution be employed, the urine will be poured from the large test-tube into the corresponding test-tube containing the Fehling's. It is convenient to have a water-bath for boiling all these tubes at one time. The rack of the water-bath should be prepared with holes to carry the desired size of tube and near each hole should be stamped a number to correspond with those on the wooden rack to prevent confusion of the tubes, according to the suggestion of Myers and Fine. The layer test may be performed by allowing the urine to run up into a piece of glass tubing of wide bore, followed by a small quantity of nitric acid, washing the tubing out after the test with water.

The results of examination may be recorded in a small pocket notebook, ruled with parallel columns in which may be recorded the names, wards, numbers of the specimens, and the findings. Tha date may be transcribed to the hospital records. The advantage of such a book is that it is convenient for making the immediate notes and it gives the person making the tests a record to which he may refer in case the report is lost or misplaced.

PREPARATION OF NORMAL SOLUTIONS.

Normal solutions are those which contain in 1 liter of water at 16° C. the hydrogen equivalent of the active reagent weighed in grams. The hydrogen equivalent of a base, acid, or salt is the molecular weight divided by the valence, the valence in the case of a base being shown by the number of hydroxyls combined with it, while that of acids is shown by the number of replaceable hydrogen atoms. For example, 1000 cc. of a normal solution of hydrochloric acid would contain 36.47 gm. of acid, since hydrochloric acid is monovalent, and the molecular weight is 36.47. Oxalic acid, however, is divalent, so one-half of its molecular weight is taken.

The following solutions are those which are used most frequently in the laboratory. The table shows the amount of the substance which should be contained in a liter of solution.

	Grams per liter.
Acetic acid	60.04
Ammonia	17.03
Ammonium sulphocyanate	76.12
Hydrochloric acid	36.47
Iodine	126.92
Oxalic acid ($COOH.COOH.2H_2O$)	63.03
Potassium hydroxide	56.11
Silver nitrate	169.89
Sodium carbonate	53.01
Sodium hydroxide	40.01
Sulphuric acid	49.04

Equal volumes of normal solution combine exactly with each other, that is, 10 cc. of normal sodium hydrate solution exactly neutralize 10 cc. of normal hydrochloric acid solution.

Seminormal, quintinormal, decinormal, vigintinormal, and centinormal solutions are used, and are designated respectively by the abbreviations $\frac{N}{2}$ or 0.5 N, $\frac{N}{5}$ or 0.2 N, $\frac{N}{10}$ or 0.1 N, $\frac{N}{20}$ or 0.05 N, $\frac{N}{100}$ or 0.01 N. They are usually prepared from corresponding normal solutions by dilution with water. For example, $\frac{N}{10}$ hydrochloric acid would be prepared from $\frac{N}{1}$ hydrochloric acid by placing 100 cc. of the latter in a 1000 cc. volumetric flask, adding enough distilled water at 16° C. to make the volume 1000 cc.

The solution most frequently required in the clinical laboratory is decinormal sodium hydrate. Unless accurate balances and volumetric glassware be available, it is better to secure this solution from a reliable chemist.

Since it is impossible to weigh sodium hydrate accurately because its weight changes due to the absorption of water, a sodium hydrate solution must be made up approximately and then titrated against an acid of known strength. An acid which is available as a standard is oxalic acid.

Preparation of Normal Oxalic Acid.—Perfect crystals of a chemically pure preparation should be selected, and 63.03 gm. weighed out on an analytical balance. The crystals are transferred to a volumetric flask and enough distilled water is added to dissolve. Distilled water is then added to bring the volume exactly to the 1000 cc. mark, the flask is stoppered, and the contents are mixed thoroughly. The accuracy of this solution may be checked by titration against normal sodium carbonate.

Preparation of Normal Sodium Hydrate.—"Sodium hydrate by alcohol" should be obtained. About 45 gm. of the sticks are weighed out and dissolved in a liter of distilled water. After the temperature has been reduced to 16° C., 50 cc. of the alkali solution are measured into a white porcelain dish. A drop of methyl orange solution is added and normal oxalic acid solution run in from a burette while stirring the mixture until the pink color becomes yellow. The reading of the burette is taken. If the sodium hydrate solution be of proper strength, 50 cc. of oxalic acid should have been used to neutralize. As made up, the solution will probably be too strong, so that less than 50 cc. of acid will be required for neutralization. When this is the case, the amount of water which must be added to the sodium hydrate may be estimated. For example, when only 45 cc. of oxalic acid solution are required, the solution is 5 per cent. too strong, and 1 cc. of distilled water should be added for each 19 cc. of solution in the flask. After the water has been added, the solution should be titrated again and if it still be too strong, the process of dilution and titration should be repeated until exactly 50 cc. of $\frac{N}{1}$ oxalic acid solution are required to neutralize 50 cc. of sodium hydrate solution. Decinormal solution may be prepared from the normal solution by placing 100 cc. of the latter in a liter volumetric flask and adding distilled water to bring the volume exactly to 1000 cc.

Preparation of Normal Sodium Carbonate.—Sutton[1] recommends the use of sodium carbonate for the preparation of the standard alkali solution against which the acid should be titrated.

A platinum crucible is half filled with powdered sodium bicarbonate. The crucible is placed in an air-bath whose temperature is 200° C. The temperature is raised to about 270° C., not over 300° C. The crucible is allowed to remain at this temperature for about half an hour, when it is removed and the sodium carbonate cooled in an exsiccator. When cool, the powder is transferred to a cool and perfectly dry bottle from which it is removed and weighed rapidly. About 2 gm. are weighed out and dissolved in 80 or 100 cc. of water. A few drops of methyl orange are added to the solution and the acid to be standardized is added from a burette until the mixture has a pure rose color. The reading is taken. It should take 100 cc. of a normal acid to neutralize 5.3 gm. of sodium carbonate. The amount of acid (X) which should have been required to neutralize the amount of sodium carbonate used may be computed as follows:

Gm. sodium carbonate: 5.3 : : cc. acid solution used : X.

For example, if 2.4 gm. of carbonate were weighed out, and 42 cc. of acid solution were employed, then the equation would be:

$$2.4 : 5.3 : : 42 : X = 92.7$$

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[1] Volumetric Analysis, Ninth Edition, 1904, P. Blakiston's Son & Co., Philadelphia.

This determination should be checked by weighing out a second quantity of sodium carbonate and repeating the titration, taking the average of the results. Should the average be 92.7, the acid solution is too strong, *i. e.*, 92.7 cc. contains as much acid as 100 cc. should, so that 900 cc. of the solution should be measured in a 1000 cc. volumetric flask, 27 cc. measured out with a burette, and water added to the liter mark. Sutton recommends preparing sulphuric acid solution standardized in this way against normal sodium carbonate solution. From the acid solution, a normal sodium hydrate solution may be made.

STAINING METHODS AND PREPARATION OF STAINS.

All of the ordinary stains are prepared very simply from the powdered dyes. It is much better for the worker to make his own stains from the dyes than to purchase them in the liquid form. Saturated alcoholic solutions may be kept on hand, and dilutions made from them as desired. The following table, taken from Wood, shows the amount of dye required to saturate 100 cc. of solvent:

Fuchsin (to saturate 100 cc. of 96 per cent. alcohol)	3.0 gm.
Gentian violet (to saturate 100 cc. of 96 per cent. alcohol)	4.8 "
Gentian violet (to saturate 100 cc. of water)	1.5 "
Methylene blue (to saturate 100 cc. of 96 per cent. alcohol)	2.0 "
Methylene blue (to saturate 100 cc. of water)	6.7 "
Wright's stain (to saturate 100 cc. of absolute methyl alcohol)	0.3 "

The majority of the saturated alcoholic solutions keep indefinitely as stock solutions. Several manufacturers offer compressed tablets of powdered stains which may be dissolved in small quantities of diluent. These are convenient when stains are not used in large quantities.

Loeffler's Methylene Blue Solution.

Saturated alcoholic solution of methylene blue	30 cc.
Potassium hydrate, 1 : 10,000 solution	100 "

Films are stained from one to five minutes. Heat is usually unnecessary but may be employed if more intense staining be desired. *Diphtheria bacilli* may be demonstrated by staining for five minutes and decolorizing with 0.5 per cent. acetic acid until the film has a gray tint.

Carbol-fuchsin (Ziehl-Neelsen Solution).

Fuchsin, saturated alcoholic solution	10 cc.
Phenol, 5 per cent. aqueous solution	90 "

Films are stained for about two minutes. The stain is heated to steaming but should not be allowed to dry. Fresh stain should be dropped on during the staining process.

Pappenheim's Decolorizing Fluid.

Rosolic acid (corallin)	1 gm.
Absolute alcohol	100 cc.
Methylene blue to saturation,	
Glycerol	20 "

The glycerol is added after the other substances have been mixed.

The decolorizing fluid is poured on a slide which has been stained with carbol-fuchsin and drained slowly, repeating the process 5 or 6 times.

Summary of Technic for Tubercle Staining.

1. Cover slide with carbol-fuchsin and steam for two minutes.
2. Wash off stain with water and place slide in acid alcohol (one part of hydrochloric acid and 99 parts of 85 per cent. alcohol) for three to five seconds, or until film shows only a faint pink color.
3. Immerse in 95 per cent. alcohol until no further color is removed.
4. Dry and stain with Loeffler's methylene blue for two minutes.
5. Dry and examine with oil immersion.

As an alternative procedure, Pappenheim's rosolic acid decolorizing fluid may be applied after the slide has been stained with carbol-fuchsin, taking the place of steps, 2, 3, and 4.

Niesser's Stain for the Diphtheria Bacillus.

Methylene blue, in powder	1 gm.
Alcohol, 96 per cent.	20 cc.
Acetic acid, glacial	50 "
Distilled water	950 "

The methylene blue is dissolved in the alcohol, and then water and glacial acetic acid are added, and the mixture filtered.

Stain for from one to three seconds, wash in water, stain with Bismarck-brown for five seconds or more, wash and dry.

See also Loeffler's methylene blue above.

Gram's Stain.—*Aniline-water Gentian Violet.*—One cc. of aniline oil is shaken up with 25 cc. of distilled water. The resulting emulsion is filtered through filter paper and used in making the stain as indicated in the formula:

Watery emulsion of anilin oil	18 cc.
Gentian violet, saturated alcoholic solution	2 "

This solution keeps only a few days. It should be filtered before using. Films are stained from one to three minutes.

Sterling's Gentian Violet Solution.

Gentian violet	5 gm.
Alcohol, 95 per cent.	10 cc.
Anilin oil	2 "
Distilled water	88 "

This solution has the advantage of lasting three or four months.

The aniline oil and alcohol are thoroughly mixed by shaking and water is added. The gentian violet is ground up in a mortar meanwhile adding the aniline solution slowly. When the maximum degree of solution has been attained, the resulting mixture is filtered. The stain is placed on the film and poured off. After one minute, the film is washed with Gram's iodine solution. It is covered with fresh iodine solution, which is allowed to remain for two minutes.

Gram's Iodine Solution.

Iodine	1 gm.
Potassium iodide	2 "
Distilled water	100 cc.

The iodine and potassium iodide are dissolved by stirring them together in a beaker with 20 cc. of water, grinding the chemicals against the wall of the beaker with a glass rod until solution takes place, when the remainder of the water is added.

This solution is used to wash off the gentian violet stain from the slide, when fresh solution is allowed to remain on the film for two minutes. It should be washed off in turn with absolute alcohol (98 per cent.). The slide is then placed in absolute alcohol (98 per cent.) for from one to ten minutes, or until no further stain is removed. Water should not be used for washing the film at any stage of the staining process. The slide is now ready for counterstaining.

Counterstains for Gram's Stain.—*Bismarck Brown.*—A saturated aqueous solution of Bismarck brown is made with the aid of heat. After cooling, it is filtered and diluted with 10 parts of water. The film may be stained from two to five minutes. There is little danger of overstaining.

Diluted Carbol-fuchsin.—This should be made by diluting 1 part of carbol-fuchsin with 9 parts of distilled water. It is allowed to remain on the slide for about two minutes.

Safranin.—A saturated solution of safranin is made with 95 per cent. alcohol. One part is diluted with 9 parts of distilled water. The stain is allowed to act for two minutes.

Summary of Technic for Gram's Stain.

1. Cover film with Sterling's gentian violet solution and allow to remain one minute.
2. Wash off with Gram's iodine solution.
3. Cover with fresh iodine solution and allow to remain two minutes.
4. Wash off with absolute alcohol.
5. Immerse in absolute alcohol from one to ten minutes, or until no further stain is removed.
6. Dry and apply counterstain.
7. Dry and examine with oil-immersion lens.

Results with Gram's Stain.—Bacteria retaining the original stain and appearing purple or lilac are said to be Gram-positive while those that are decolorized, losing the gentian violet and taking the counterstain, are Gram-negative.

Classification of important pathogenic bacteria according to Gram's stain:

Gram Positive (Retain the violet color)

- Bacillus aërogenes capsulatus
- Bacillus anthracis
- Bacillus botulinus
- Bacillus diphtheriæ
- Bacillus subtilis
- Bacillus tetanus
- Bacillus tuberculosis and other acid-fast bacilli
- Diplococcus pneumoniæ
- Micrococcus pyogenes
- Micrococcus pyogenes albus
- Micrococcus pyogenes aureus

Gram Negative (Lose the violet color and take the counterstain)

- Bacillus coli
- Bacillus dysenteriæ
- Bacillus influenzæ
- Bacillus Koch-Weeks
- Bacillus mallei
- Bacillus Morax-Axenfeld
- Bacillus mucosus capsulatus
- Bacillus paratyphosus
- Bacillus pestis
- Bacillus proteus
- Bacillus pyocyaneus
- Bacillus typhosus
- Diplococcus gonorrhea
- Diplococcus intracellularis meningitidis
- Micrococcus catarrhalis
- Spirillum choleræ

Capsule Stains.—*Hiss' Capsule Stain.*—Preparations are made on slides or cover-slips, preferably from animal exudates. If preparations be made from cultures, a drop of fresh blood-serum should be added to the drop of culture material in making the film. Water must not be used at any stage of the procedure. The film is dried in the air, carefully fixed with heat, and flooded with a solution of gentian violet (5 cc. of saturated alcoholic solution diluted with 95 cc. of distilled water). The stain is steamed over a free flame for a few seconds, when it is washed off with a 20 per cent. solution of copper sulphate. This is poured off and the slide dried and mounted in balsam. The capsules appear as fainty blue haloes around dark purple cell-bodies. Avery and co-workers state that better results may be obtained by omitting heat fixation altogether, and by washing off the gentian violet solution with copper sulphate as soon as it begins to steam.

Welch's Capsule Stain.—A cover-slip preparation, made in the usual way, is allowed to dry in the air without the aid of heat, and covered with glacial acetic acid for a few seconds. The acid is drained and the film covered with anilin-water gentian violet. This is poured off and replaced by fresh stain, repeating the process several times. The stain is left on for three or four minutes the last time and then washed off in 2 per cent. sodium chloride solution. The cover-slip is mounted without drying on a slide in a few drops of the salt solution and examined with the oil-immersion lens. Water should not be used in making the preparation or during the process of staining.

Staining for Spores.—Special methods are necessary to penetrate the resistant spore membrane. The film may be stained by steaming with carbol-fuchsin for two minutes, washing with water, and then decolorizing with a 1 per cent. dilution of sulphuric acid in water. Counter-staining is accomplished with Loeffler's methylene blue. This is merely an adaptation of the method used for tubercle bacilli. The bodies of the bacteria stain blue, the spores are red. If the spores are too resistant to take up the stain with this method, either Moeller's or Huntoon's method may be employed.

Moeller's Method.—A thin film is dried, fixed with heat, washed in chloroform for two minutes, then in water, and placed in 5 per cent. chromic acid for three minutes. This is washed off with water and the film covered with carbol-fuchsin, which is steamed for two minutes and washed off with water. Five per cent. sulphuric acid is used for decolorizing and methylene blue applied for two minutes as a counterstain. The spores are red and the bodies of the bacteria blue.

Huntoon's Method.—Two solutions are needed.

A.

Acid fuchsin	4 gm.
Acetic acid, 2 per cent. aqueous solution	50 cc.

B.

Methylene blue	2 gm.
Acetic acid, 2 per cent. aqueous solution	50 cc.

The two solutions are mixed and allowed to stand for fifteen minutes. The heavy precipitate which ensues is removed by filtration and the filtrate used for staining. The stain keeps for several weeks.

For staining, a rather thick film is covered with the stain for one minute. The bright-red film is dipped in a dilute solution of sodium carbonate (7 or 8 drops of a saturated solution in 250 cc. of water) until it turns blue, when it is removed, washed with water, dried, and examined with the oil-immersion lens. The spores stain red and the bodies of the bacilli blue.

Staining Flagella.—(Bunge's modification of Loeffler's method).—The procedure is difficult and requires strict adherence to detail. Cultures must be employed. To 2 or 3 cc. of distilled water in a test-tube is added enough growth from a young (twelve to eighteen hours) agar culture to produce slight cloudiness. The suspension may be placed in the incubator at 37° C. for a few hours to permit some development. A small drop of the suspension is spread upon a thoroughly cleaned cover-slip as a very thin film in order to secure quick drying.[1] The film is fixed by passing the cover-slip through the flame three times, and then covered with the mordant, which is allowed to act without heat for five minutes when it is warmed for one minute and washed off. After drying, it is stained with anilin-water gentian violet, warming gently for about a minute. The staining process may have to be repeated a number of times. The mordant is prepared according to the following formula:

Tannin, saturated water solution	3 parts
Ferric chloride, 25 per cent. watery solution	1 part

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[1] See method for cleaning cover-slips described for making blood films, page 44.

This must be allowed to stand several weeks before using.

Staining Treponemata.—Methods of demonstrating treponemata by staining and dark-field illumination have been described on page 397.

Wright's Stain.—The technic for staining with Wright's stain has been given fully in the chapter on the Examination of Blood. The stain may be prepared from the powder by dissolving 0.1 gm. in 60 cc. of methyl alcohol (preferably Merck's reagent). The powder should be rubbed up in a mortar with pestle to ensure thorough solution. It should be filtered. It is important to have all apparatus free from acid.

The stain may be prepared from methylene blue and eosin as follows:

Methylene blue (B. X. or medicinal) is added to 0.5 solution of sodium bicarbonate in the proportion of 1 gm. of dye to each 100 cc. of solution. The mixture is placed in a flask or flasks so that the layers of fluid will not be more than 6 cm. deep and heated in a steam sterilizer at 100° C. for a full hour. It is then cooled by placing the flasks in cold water when the precipitate may be removed by filtration. When cool, the solution should have a deep purple-red color when a thin layer is viewed with transmitted yellow artificial light.

To each 100 cc. of the filtrate are added 500 cc. of a 0.1 per cent. aqueous solution of "yellowish, water-soluble" eosin. After thorough mixing, the heavy precipitate which forms is collected on a filter and dried. The powder may be put away and dissolved with methyl alcohol as desired. The proportions are given above.

Wilson's Stain.—Silver oxide is freshly precipitated by dissolving 2 gm. silver nitrate in 15 cc. of distilled water and adding 260 cc. of freshly prepared solution of calcium hydrate.[1] The brown precipitate of silver oxide is collected on a coarse filter paper, washed with water, and dried at a temperature not exceeding 80° C., when it is used in preparing solution A.

Two solutions are made:

Solution A.

Methylene blue	2 gm.
Sodium bicarbonate	1 "
Silver oxide	2 "
Distilled water	200 cc.

Solution B.

Eosin (yellow, water soluble)	1 gm.
Distilled water	200 cc.

Solution A is boiled slowly in a deep porcelain dish, stirring occasionally for twenty minutes when about 65 cc. are poured into a graduated cylinder, and an equal quantity of water added to the boiling solution. In twenty minutes about one-half the quantity of fluid in the porcelain dish is again poured into the graduate and boiling continued for twenty minutes. The solution is added to the other two fractions in the graduate and enough distilled water added to bring the total volume to 200 cc. The solution is immediately filtered through coarse filter paper and Solution B added. The two fluids are mixed thoroughly and at the end of fifteen to thirty minutes the mixture is filtered through hard filter paper to secure the brownish-red precipitate. During filtration the sides of the filter should be washed down with normal sodium chloride. The precipitate may be dried in the air, by suction, or preferably in a hot air sterilizer at a temperature not in excess of 60 to 80° C. The powder is bluish-black in color with green luster. It should be kept in a dark bottle away from the light. To make staining fluid, 0.4 gm. of the powder are dissolved in 100 cc. absolute methyl alcohol.

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[1] Calcium hydrate may be prepared by dissolving a small quantity of unslaked lime in 2 or 3 liters of water, stirring well, and allowing to settle, when the supernatant layer is siphoned off to remove the impurities and fresh water is added. The washing process is repeated 2 or 3 times and the fluid from the third washing used in preparing the silver oxide.

Staining is carried out as with Wright's stain. The film is dried in the air and covered with stain. After a minute, an equal quantity of water is added. The diluted stain is allowed to act for four minutes when it is washed off with water until the film shows a pinkish-brown or tan shade. The film is dried and mounted in balsam.

Giemsa Stain.

Azur II eosin	3.0 gm.
Azur II	0.8 "
Glycerol (C. P.)	250.0 cc.
Methyl alcohol (H. P.)	250.0 "

For staining, fresh dilution is prepared by adding 1 drop to 10 cc. of distilled water. The preparation is dried in the air, fixed with methyl alcohol for two minutes, and covered with *diluted* stain. At the end of ten or fifteen minutes, this is washed off. The film is dried and mounted in balsam. Staining may be intensified by adding 1 or 2 drops of 1 per cent. sodium carbonate to 10 cc. of diluted stain.

Jenner Stain.

Eosin (Grubler's water soluble) 1.2 per cent. solution in distilled water	100 cc.
Methylene blue (Grubler's medicinal) 1 per cent. solution in distilled water	100 "

The solutions are stirred together in a beaker. The mixture is allowed to stand twenty-four hours, when it is filtered through paper to secure the precipitate, which is washed by pouring distilled water through the filter until the filtrate shows only a slight blue color. The precipitate is then scraped from the filter and dried *in the air*, without heat. 0.5 gm. of dry powder are dissolved in 100 cc. of methyl alcohol (H. P.). A blood film, dried in the air, is covered with the stain. After one to three minutes, it is rinsed with distilled water until the film is pale pink.

Antiformin.—Antiformin may be prepared according to this formula:

Sodium hydrate	7.5 gm.
Liquor sodæ chlorinatæ (Labarraque's solution)	100.0 cc.

Liquor sodæ chlorinatæ may be made according to the following directions after the U. S. pharmacopœia, although Stitt recommends doubling the quantities of sodium carbonate and chlorinated lime.

Liquor Sodæ Chlorinatæ.

Sodium carbonate (monohydrated)	70 gm.
Chlorinated lime	100 "
Water, to make	1000 "

Dissolve sodium carbonate in 500 cc. of hot water, and add it to a previously prepared magma of chlorinated lime, made by rubbing up the latter with 500 cc. of water. Shake the mixture well and warm if it gelatinizes. Filter through a moistened muslin strainer, returning the first of the filtrate through the strainer. Make up to 1000 gm. Keep in a dark cool place in well stoppered bottles.

PURIFICATION OF PICRIC ACID.

Picric acid may not be chemically pure even when so labeled. The following method has been recommended for determining its purity: A saturated solution is made in water. Some of this is placed in the left hand cup of the colorimeter. Ten cc. are placed in a tube or graduate and to it is added 0.5 cc. of 10 per cent. sodium hydrate solution. This is allowed to stand for ten minutes and is then placed in the right hand cup of the colorimeter. The left hand cup should be placed at 20 mm. When the color is matched exactly, the reading of the right hand cup should not be less than 15 mm. In other words, the picric acid should not show more than 25 per cent. reaction.

Purification of picric acid may be carried on according to the method of Folin and Doisy. Six hundred grams of the wet picric acid are transferred to a large beaker or a large porcelain casserole, holding not less than 4 liters. Boiling water is added until the beaker is nearly full. In this should be placed 200 cc. of 50 per cent. sodium hydrate. The mixture is stirred and heated gently, if this be necessary, to dissolve all of the acid. While the mixture is still hot, 200 gm. of sodium chloride should be stirred in slowly. The beaker is then cooled until the temperature is about 30° C., stirring occasionally. Then the contents are filtered through a large Buchner funnel and the sediment is washed a few times with 5 per cent. sodium chloride. The sediment is then transferred to a large beaker which is filled with boiling water. When the picrate has been dissolved, 50 cc. of 10 per cent. sodium hydrate should be added with stirring, and then 100 gm. of sodium chloride. This is cooled to 30° C. with stirring, filtered and washed with 5 per cent. sodium chloride solution as before. The solution and precipitation of the sodium picrate is repeated twice more. For the last washing, the pure water is used instead of the sodium chloride.

The purified picrate is then dissolved in a large beaker with boiling distilled water and is filtered while still hot through a large folded filter, collecting the filtrate in a large flask. To the hot filtrate is added 100 cc. concentrated sulphuric acid which has been previously diluted with 2 volumes of distilled water. The liberated picric acid will begin to crystallize almost at once. A beaker is placed over the mouth of the flask and the flask is cooled under running water to about 30° C. This is filtered through a Buchner filter and is washed free from sulphate with distilled water.

PREPARATION OF CREATININE ZINC CHLORIDE.

Creatinine zinc chloride may be readily prepared by Benedict's modification of Folin's method. To about 10 liters of undecomposed urine are added 180 gm. of picric acid. The acid should be dissolved first in boiling alcohol, using approximately 100 cc. of alcohol for each 40 gm. of picric acid. This hot alcoholic solution of picric acid should be poured into the urine while the latter is being stirred vigorously. After the mixture has stood over night in a precipitating jar, the supernatant fluid may be siphoned off. The residue is poured upon a large Buchner funnel and is drained with suction. After draining, it should be washed once or twice with a cold, saturated solution of picric acid and sucked dry by the vacuum. The dry, or nearly dry, precipitate should be placed in a large mortar or evaporating dish with sufficient concentrated hydrochloric acid to form a moderately thin paste. The proper amount is usually 60 cc. of acid for 100 gm. of picrate. This mixture is stirred thoroughly with a pestle for from three to five minutes, when it is filtered with suction through a hard filter paper, washing the residue with twice enough water to cover it. The washing process should be repeated twice when the filtrate may be transferred to a large flask and neutralized with an excess of solid magnesium oxide, the variety is known commercially as the heavy magnesium oxide. The oxide should be added in small portions. When the hydrochloric acid has been completely neutralized, the mixture turns a bright lemon-yellow. Litmus may be used to determine the end-point of neutralization. This mixture is filtered with suction and the precipitate is washed two or three times with water. The residue is discarded and the filtrate is treated with sufficient glacial acetic acid to strongly acidify it. A few cc. of acid are usually sufficient for this purpose. No attention need be paid to a precipitate which may form at this point. The solution should then be diluted with about 4 volumes of 95 per cent. alcohol and filtered with suction after it has been allowed to stand for fifteen minutes. Filtration is necessary to remove the slight precipitate which then forms, composed chiefly of calcium sulphate. The filtrate finally obtained is treated with 30 per cent. solution of zinc chloride. Three or four cc. of zinc chloride solution should be used to each liter of urine originally employed. The mixture is stirred and allowed to stand over night in a cool place. A precipitate should form almost at once. After standing over night, the supernatant fluid is poured off and the precipitate, creatinine zinc chloride, is collected on a Buchner funnel, washed with water, next with 50 per cent. alcohol, then with 95 per cent. alcohol, and finally dried. The substance obtained should be a nearly white crystalline powder. Standard creatinine solution may be conveniently prepared from the creatinine zinc chloride by weighing out 1.6106 gm. of the thoroughly dried salt and dissolving it in sufficient decinormal hydrochloric acid to make 1 liter. Such a solution contains 1 mg. of creatinine per cc. From this one may readily prepare stock solutions for the three standards for blood determination which will contain 0.3, 0.5 and 0.1 mg. of creatinine per 100 cc. of saturated solution of picric acid by placing in 100 cc. volumetric flask 0.3 cc., 0.5 cc. and 1.0 cc. of the standard solution and bringing to volume with saturated solution of picric acid. The standard for determinations in the urine is prepared each time by placing 1 cc. of the standard (containing 1 mg. per cc.) in a 100 cc. volumetric flask, and adding 20 cc. of saturated solution of picric acid, 1.5 cc. of 10 per cent. sodium hydrate, and water to 100 cc.

APPARATUS.

The first list includes apparatus required to carry out the simple routine tests. That required for more complicated procedures, such as complement-fixation tests or blood chemistry, is given in special lists which follow. Mention of apparatus required for other less frequently employed methods will be found in the text. Bacteriological apparatus is described in the chapter on Bacteriological Methods. All the lists are subject to adaptations and substitutions. The number of pieces of the different articles has not been given, because this will depend entirely upon the number of specimens which are to be examined and the capacity of the laboratory. The reagents for all methods have been given with the description of the methods, since this seemed to be the more convenient arrangement.

Minimum Equipment for Clinical Laboratory.

Alcohol lamp.

Bacteriological loop.

Beakers, about 50 cc. and 100 cc. capacity, with lip.

Blood lancet.

Bottles, one ounce capacity, to contain solutions for blood counting, *i. e.*, alcohol, ether, distilled water, Hayem's solution, 0.5 per cent. acetic acid, $\frac{N}{10}$ HCl.

Burner, Bunsen, Tyrrill, or micro type.

Burette, 25 or 50 cc. capacity, graduated in 0.1 cc., with glass stopcock or rubber tubing outlet and Mohr pinchcock.

Burette stand for supporting burette.

Camel's-hair brush.

Centrifuge, water, hand, or electric power. The last form is preferable because cleanest and most efficient.

Conical glasses for receiving specimens of urine.

Cover-glasses, one inch square, No. 1 thickness.

Cover-glass forceps, Stewart's type.

Dropping bottles, convenient for stains and indicators.

Erlenmeyer flasks, 100, 250, and 500 cc. capacity.

Esbach albuminometer.

Evaporating dish, smooth inner surface, white porcelain, about 10 cm. diameter.

Filter papers, in packs, about 12 cm. diameter.

Funnels, glass, about 5 and 12 cm. diameter.

Glass tubing, about 2 kg. in assorted sizes. Convenient for making pipettes, etc.

Graduates, 10, 25, and 100 cc. capacity.

Hemocytometer. The best form has been described in the text.

Hemoglobinometer. Preferably Sahli's though the other forms described in the text may be used.

Labels, for object slides and for bottles.

Lens paper.

Mechanical stage.

Medicine droppers.

Microscope. If a Bausch and Lomb be purchased, suitable combination of lens includes 5 and 10 oculars, 4 mm. and 16 mm. dry objectives, and 1.9 mm. oil-immersion objective mounted on a revolving nosepiece. It should be fitted with an Abbé substage condenser, and iris diaphragms for stage and condenser.

Mortar, porcelain, about 8 cm. diameter, and pestle.

Object slides, 1 by 3 inches, or good quality glass, preferably with polished edges.

Object slide box.

Pencil, for writing on glass, Blaisdell's No. 163, China marking.

Petri dishes with covers.

Pipettes, 5 and 10 cc. capacity, one mark only; 1 cc. capacity, graduated in 0.1 cc.

Ring stand for boiling beakers, etc.

Stirring rod, glass.

Stomach tube, either Ewald or Rehfuss. Latter is required if duodenal contents are to be obtained. Pollitzer bulb to fit stomach tube.

Glass syringe to fit Rehfuss tube.

Test-tubes, glass, about 16 by 150 mm., with flange.

Test-tube clamp, wooden.

Test-tube rack, wooden, with 6 pegs and 6 holes.

Urinometer.

Apparatus required for blood chemistry and quantitative determinations on urine (Fig. 54):

Aërating apparatus.—This may be improvised. (See Fig. 55.)

Analytical balances.

Burette, 50 cc. capacity, graduated in 0.1 cc.

Beakers, 100, 250, 500, 1000 cc. capacity.

Centrifuge, preferably a heavy one which will take 50 cc. centrifuge tubes (Fig. 57), although a smaller centrifuge may be used.

Centrifuge tubes, 50 cc., round bottom, with pour-out.

Centrifuge tubes, 50 cc., graduated, with pour-out.

Centrifuge tubes, 15 cc., graduated, if small centrifuge be used.

Colorimeter.

Filter papers, fat-free, about 6 cm. diameter.

Flasks; Erlenmeyer pattern, 100, 250, 500 cc. capacity; Florence pattern, 500 cc., 1 and 2 liter capacity.

Folin absorption tubes. Folin absorption tube may be made from a piece of glass tubing. One end is drawn out to a fine point as though a capillary pipette were to be made. This is broken off and sealed in the flame and then softened thoroughly by heating. Air is blown into the closed tube from the other end until the heated end bulges to form a small bulb. A platinum or nichrome wire (mounted in a handle) is heated red hot. One side of the glass bulb is heated in the edge of the flame to soften an area through which the hot wire is plunged. Three or four holes are made in this way.

Folin's blood sugar tubes (Fig. 54, *m*).

Glass beakers, 250 and 500 cc. capacity.

Glass funnels, about 5 cm. and about 15 cm. diameter.

Glass stirring rods.

Graduates, cylindrical, accurately graduated, of 10, 25, 50, and 100 cc. capacity.

Ignition tubes of Pyrex glass, 20 to 25 mm. by 200 mm. Folin's tubes are of advantage (Fig. 54, *k*). They are graduated at 35 and 50 cc. They may be obtained from the Emil Greiner Co.

Micro burner.

Myers' blood sugar tubes (Fig. 54, *l*).

Pipettes.—(*a*) Ordinary Mohr pipettes with one mark only, of 1, 2, 3, 4, 5, 10, 20, and 50 cc. capacity (Fig. 54, *b*).
- (*b*) Ostwald pipettes, of 1, 2, 3, 5, and 10 cc. capacity (Fig. 54, *d*).
- (*c*) Folin pipettes (Fig. 54, *a*).
- (*d*) Mohr pipettes, graduated, capacity 1 and 2 cc., graduated in one-hundredths cc. (Fig. 54, *e*).

Quartz pebbles.

Test-tube rack.

Tripod and clamp to hold test-tube over burner.

Volumetric flasks, 10, 20, 25, 50, 60, 100, 500, 1000 cc. capacity (Fig. 54, *j*).

For quantitative determinations on the urine, the same apparatus will be sufficient with the following additions:

Kjeldahl flasks.

Kjeldahl distilling apparatus. When many determinations are to be made at one time, a special apparatus (Fig. 72) should be obtained. If only an occasional specimen be examined, a distilling apparatus may be improvised on the principle of a Liebig condenser (Fig. 71).

Apparatus Required for Serology (Fig. 32):

Animal cages are convenient but wooden boxes may be employed.

Apparatus for taking blood, MacRae's or the author's (Fig. 32, *d* and *e*).

Balances, platform, for balancing loaded tubes before placing in centrifuge.

Centrifuge. While a small, rapid electric centrifuge may be used, a heavy centrifuge fitted with two interchangeable heads for 15 and 50 cc. tubes, is désirable (Fig. 57).

Centrifuge tubes, graduates 15 cc. tubes with conical tip, and 50 cc. tubes with round bottom.

Erlenmeyer flasks, 250, 500 and 1000 cc. capacity.

Files, triangular.

Funnels, glass, diameter, 10 to 15 cm.

Graduates, 100 and 200 cc. capacity. Those with ground glass stoppers are convenient for diluting and storing reagents during day's work (Fig. 32, *c*).

Knives, long amputating or autopsy knife, and small scalpel.

Pipettes boxes, cylindrical, of copper.

Pipettes, capillary. These are easily prepared from 6 inch pieces of clean, dry glass tubing, which may be held over a Bunsen flame until *thoroughly softened,* and then removed and pulled out until a capillary portion of the desired size is obtained. This is allowed to cool, when it may be broken to form two pipettes.

Pipettes, volumetric (Fig. 32, *a* and *b*).

- 1 cc. capacity, graduated in 0.1 cc.
- 1 cc. capacity, graduates in 0.01 cc.
- 5 cc. capacity, graduated in 0.05 cc.
- 10 cc. capacity, graduated in 0.1 cc.

Refrigerator.

Syringes, all glass, Luer type, 2 cc. and 5 cc. capacity.

Sterilizer, dry air, Lautenschlager type.

Still, copper with worm condenser, or Barnstead.

Test-tubes. For the technic described here, employing a bulk of 2.5 cc. in the completed reaction, a tube about $\frac{5}{8}$ by 4 inches should be used. Tubes of unusually resistant glass should be used. These may be obtained from the Steele Glass Co., Locust St., Philadelphia.

Test-tube racks, of metal, to fit water-bath (Fig. 35).

Water-bath or incubator, maintained at temperature of 37° C. If only a small equipment is purchased, the bacteriological dry air incubator may be used, but longer incubation will have to be employed. The water-bath should be deep enough to permit immersion of the tubes to one-half their depth, and is heated preferably with electricity, automatically controlled (Fig. 36).

Water-bath maintained at a temperature of 56° C. A simple water-bath may be used, or it may be fitted with a thermoregulatory device.

Wire baskets for holding test-tubes,

CARE OF GLASSWARE FOR SEROLOGICAL WORK.

It is absolutely essential that all glassware be chemically clean. Bacteriological sterility is desirable but not absolutely necessary except in securing and handling blood which is to be preserved as amboceptor, or specimens of human blood which are to be held some days before examination.

Test-tubes employed in the reaction should be emptied immediately after use. If the process of cleaning can be finished at once, a test-tube brush should be used to remove any sediment of blood, the tubes rinsed with several changes of tap water, then with distilled water and finally placed upside down in wire baskets and put in dry-air sterilizer for an hour at 200° C. When cleaning cannot be finished at once. the contents of the tubes should be poured out and the tubes then put to soak in clear water until the foregoing procedure can be carried out. Blood clots should be shaken out as soon as the reactions are completed, the tube brushed vigorously a number of times with a test-tube brush, rinsed with several changes of clear water and then placed in clear water for several hours when the cleaning may be completed as previously outlined. *Flasks and graduated cylinders* may be cleaned with repeated changes of water and rinsed with distilled water. Brushing is rarely necessary. *Pipettes* should be given scrupulous care. Immediately after use they should be placed in tall cylinders filled with distilled water in order to prevent drying and coagulation of the serum. Later the cotton plugs may be extracted with a barbed dental broach (Fig. 32, *h*). Then the upper end of each pipette in turn should be inserted into the rubber-tubing outlet of a reservoir bottle of distilled water and the water should be allowed to flow through. It is advisable to soak the pipettes after this in a flat enamel basin with distilled water, and to repeat the process or running distilled water through in twenty-four hours. When thoroughly clean the pipettes may be dried in the oven and the upper ends then lightly packed with a little cotton (Fig. 32, *a* and *b*). *Capillary pipettes* are discarded after using once. It is a waste of time to attempt to clean them properly.

Glassware should always be transparent, bright, shiny, and free from the evidence of dried serum or blood. This is easy to accomplish if the simple methods of cleaning just outlined are employed. *The routine use of chemical cleaning fluids is both unnecessary and unwise* on account of the possibility of insufficient rinsing. Dependence should be placed upon brushing, wiping, and the copious use of water. When tubes become smoky and dried serum occludes the pipettes, the glassware may be boiled for thirty minutes in 2 to 5 per cent. sodium carbonate, rinsed in clear water until litmus paper fails to show any trace of alkali, and finally cleaned by the use of distilled water and dried in the hot air sterilizer.

PREPARATION OF AUTOGENOUS VACCINES.

Preparation of the Emulsion.—The organisms are isolated and obtained in pure culture in the usual way. This should be accomplished as rapidly as possible. When a pure culture is obtained, several tubes of solid medium should be planted, selecting a type of medium most suitable for the growth of the organism. The cultures are allowed to grow for twenty-four hours at 37° C., when the tubes are filled with sufficient sterile normal salt solution to entirely submerse the slanted medium. A platinum wire, mounted on a glass rod, is bent into a wide loop. This is sterilized in the Bunsen flame. After it has cooled, it is used to scrape the bacterial growth off the slanted medium and to mix it with the salt solution. The bacterial emulsion thus prepared is poured from all the tubes into a dry sterile test-tube or bottle, containing a few bits of broken glass. The ends of the tubes should be passed through the flame just before the emulsion is poured out, in order to avoid contaminating the emulsion. If a test-tube is used, it should be hermetically sealed with a blow pipe, while a bottle may be stoppered with a sterile rubber stopper or tight-fitting glass stopper. The emulsion is then shaken vigorously for fifteen to twenty minutes. A shaking machine expedites this part of the preparatory labor. The vaccine is now ready for counting.

Standardization According to a Modified Wright's Method.—The worker should provide himself with a capillary pipette the capillary portion of which should be at least six inches long and of fairly uniform bore. A mark is made at the capillary portion of the pipette about 1 cm. from the end with ink or a grease pencil and the larger end of the pipette is fitted with a rubber nipple. Such a pipette may be readily drawn from a piece of glass tubing.

The vaccine should be thoroughly shaken before counting. Two or three drops of well-shaken emulsion are placed in a watch-crystal. The worker then pricks his finger and allows a drop of blood to fall on a slide. This blood is drawn up into the pipette to the mark. After having thus measured off a quantity of blood, the column of blood is aspirated carefully up the pipette so that its base will occupy a position about 5 mm. above the tip of the pipette, and the bacterial emulsion is drawn up to the mark. In the pipette there now should be a column of blood and a column of bacterial emulsion of equal height separated by an air space about 5 mm. long. The contents of the pipette are carefully ejected onto a clean slide and are then drawn back and forth into and out of the pipette three or four times to mix the blood and the organisms thoroughly. It is desirable to avoid the admixture of air bubbles. The drop is then spread on the slide in the manner described for making blood smears, stained with Wright's stain, and counted. In a number of different fields, selected at random, the number of red blood cells and organisms are enumerated to determine their ratio. At least 1000 red cells should be counted. The preparation should be discarded and another made if the bacteria prove to be unevenly distributed. In making the count, an Ehrlich's ocular is of the utmost convenience, since the visible field may be greatly reduced, making the operation of counting much less trying. If this instrument is not available, a substitute may be made by cutting a circular piece of paper to rest on the platform which is found between the upper and lower lenses of all oculars, and by making a small hole in center.

Calculation.—Assume that the worker's blood be normal, containing five million red blood corpuscles per cm. and that equal quantities of blood and bacteria suspension were taken. The number per cm. is computed by use of this equation, in which A = the number of bacteria counted, B = the number of corpuscles counted, and X represents the number of organisms per cm.:—

$$\frac{5,000,000 \times A}{B} = X$$

Standardization According to Hopkins' Method.—The writer much prefers Hopkins' method. It requires a rapid centrifuge and an specially graduated centrifuge tube (Hopkins' tube)[1] (Fig. 136).

The bacterial emulsion is prepared as directed in the preceding method and is poured into the Hopkin's tube which is centrifugalized for thirty minutes at a rate of 2800 revolutions per second in a centrifuge whose head has a diameter of 18 cm. from the tip of one tube to the tip of the other. Then the supernatant salt is removed carefully with bacteria to reduce the top of the sediment to the mark desired. If the mark be .05, fresh salt solution may be added to the mark 1.0 and a 0.5 per cent. dilution is obtained. If the bacterial sediment comes up to the mark 0.1 and salt solution is added to the mark 5, we have a 2 per cent. dilution. Hopkins has prepared a table showing the number of bacteria represented in a 1 per cent. dilution by the different forms.

	Per cent.	Billion per cc.
Staphylococcus aureus and albus	1	10
Streptococcus hæmolyticus	1	8
Gonococcus	1	8
Pneumococcus	1	2
Bacillus typhosus	1	8
Bacillus coli	1	4

When the salt has been added to make an emulsion of the desired strength, the vaccine is poured in a sterile bottle or test-tube and is ready for sterilization.

Sterilization of the Vaccine.—The bottle or tube containing the vaccine is placed in a water-bath, and the water is kept at a temperature of 65° C. for one hour. At the end of that time, cultures are made from the vaccine on two or more tubes of the same sort of medium as was employed for growing the cultures. These cultures are allowed to incubate at least twenty-four hours. If sterile, the vaccine may be employed.

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[1] These may be obtained from the International Equipment Co., Cambridge, Mass.

Dilution and Preservation of Vaccines.—If the sterilized suspension be stronger than desired for administration, the vaccine should be diluted with sterile salt solution so that 0.1 cc. of the dilution contain the initial dose. Suppose, for example, that the sterilized and standardized emulsion is found to contain 500,000,000 bacteria per cc., and that the desired initial dose, which we wish to have in bulk of 0.1 cc., is 50,000,000, we would take 1 cc. of the emulsion and add 9 cc. of sterile salt solution.

Lysol or tricresol is added as a preservative, 3 drops being added with a sterile dropper to each 10 cc. of completed vaccine.

WEIGHTS AND MEASURES.

METRIC SYSTEM.

Meter (unit of length):
Millimeter (mm.) = 1/1000 meter
Centimeter (cm.) = 1/100 "
Kilometer = 1000 "
Micron = 1/1000 millimeter
Gram (unit of weight) (gm.):
Milligram (mg.) = 1/1000 gram
Kilogram (kg.) = 1000 "
Liter (unit of capacity) (L.):
Cubic centimeter = 1/1000 liter
[same as milliliter (ml)]

APOTHECARIES' OR TROY WEIGHT.

(Used in Prescriptions)

Grain (gr.):
[Scruple (℈) = 20 grs.]
Dram (ʒ) [= 3℈] = 60 grs.
Troy ounce (℥) = 8ʒ = 480 grs.
[Troy pound. = 12℥ = 5760 grs.]
(℥j of water under standard conditions measures 504.83 minims.)

AVOIRDUPOIS WEIGHT.

(A System Used in Commerce)

Grain = same as Troy grain
Ounce (oz.) = 437½ grains
Pound (lb.) = 16 ozs. = 7000 grains
Ton = 2000 lbs.

UNITED STATES APOTHECARIES' OR WINE MEASURE.

(Used in United States for Both Prescription and Commercial Purposes)

Minim (♏) (approximately equal to 1 drop or to 1 grain of water—more exactly, 0.95 grain)
Fluidrachm (flʒ) = 60 ♏
Fluidounce (fl℥) = 8 flʒ = 480 ♏ (fl℥j of water under standard conditions weighs 456⅖ grains).
Pint (pt., or Octarius, O) = 16 fl℥ = 7680 ♏
Quart (qt.) = 2 pts. = 32 fl℥
Gallon (gal., or Congius, C) = 8 O = 128 fl℥ = 61,440 ♏
A gallon holds 231 cubic inches.

EQUIVALENTS OF METRIC AND COMMON SYSTEMS.

Space.

1 meter	=	39.370 inches	1 inch	=	0.0254 M. = 2.54 cm.
	=	3. ft. 3.370 inches	1 ft.	=	30.227 cm.
	=	1 yd. 3.370 inches	1 yd.	=	91.440 cm.

Capacity (United States)

1 cc.	=	16.23 ♏	1 ♏	=	0.06161 cc.
1 l.	=	33.815 fl℥	1 flʒ	=	3.7 cc.
	=	2.113 pts.	1 fl℥	=	29.574 cc.
	=	0.2642 gal.	1 pt.	=	0.4731 l.
			1 gal.	=	3.7854 l.

Capacity (British)

1 l.	=	1.760 pints	1 pint	=	0.5679 l.
	=	0.2209 gallons	1 gallon	=	4.5435 l.

Weight.

1 mg.	=	$\frac{1}{64}$ gr.	1 gr.	=	64.8 mg. = 0.0648 gm.
1 gm.	=	15.432 grs.	1 ʒ	=	4 gm.
	=	0.03527 oz. Av.	1 oz. Av.	=	28.3495 gm.
	=	0.03215 ℥ Troy	1 ℥ Troy	=	31.1035 gm.
1 kg.	=	2.2046 lbs.	1 lb.	=	0.4536 kg.

Approximate Equivalents.

1 gm. 1 cc.	=	15.5 grs. or minims	1 gr. or minim	=	0 065 gm. or cc.
			1 dram	=	4.0 cc. (—)
1 mg.	=	$\frac{1}{64}$ grain	1 ounce	=	30.0 cc. (—)
1 liter	=	1 quart (+)	1 pint	=	0.5 liter (—)
1 kg.	=	2.2 lbs.			

CONVERSION OF CENTRIGRADE TO FAHRENHEIT DEGREES

°C.	°F.	°C.	°F.	°C.	°F.	°C.	°F.	°C.	°F.
−30	−22.0	+27	+80.6	+83	+181.4	+139	+282.2	+195	+383.0
−29	−20.2	28	82.4	84	183.2	140	284.0	196	384.8
−28	−18.4	29	84.2	85	185.0	141	285.8	197	386.6
−27	−16.6	30	86.0	86	186.8	142	287.6	198	388.4
−26	−14.8	31	87.8	87	188.6	143	289.4	199	390.2
−25	−13.0	32	89.6	88	190.4	144	291.2	200	392.0
−24	−11.2	33	91.4	89	192.2	145	293.0	201	393.8
−23	−9.4	34	93.2	90	194.0	146	294.8	202	395.6
−22	−7.6	35	9..0	91	195.8	147	296.6	203	397.4
−21	−5.8	36	96.8	92	197.6	148	298.4	204	399.2
−20	−4.0	37	98.6	93	199.4	149	300.2	205	401.0
−19	−2.2	38	100.4	94	201.2	150	302.0	206	402.8
−18	0.4	39	102.2	95	203.0	151	303.8	207	404.6
−17	+1.4	40	104.0	96	204.8	152	305.6	208	406.4
−16	3.2	41	105.8	97	206.6	153	307.4	209	408.2
−15	5.0	42	107.6	98	208.4	154	309.2	210	410.0
−14	6.8	43	109.4	99	210.2	155	311.0	211	411.8
−13	8.6	44	111.2	100	212.0	156	312.8	212	413.6
−12	10.4	45	113.0	101	213.8	157	314.6	213	415.4
−11	12.2	46	114.8	102	215.6	158	316.4	214	417.2
−10	14.0	47	116.6	103	217.4	159	318.2	215	419.0
−9	15.8	48	118.4	104	219.2	160	320.0	216	420.8
−8	17.6	49	120.2	105	221.0	161	321.8	217	422.6
−7	19.4	50	122.0	106	222.8	162	323.6	218	424.4
−6	21.2	51	123.8	107	224.6	163	325.4	219	426.2
−5	23.0	52	125.6	108	226.4	164	327.2	220	428.0
−4	24.8	53	127.4	109	228.2	165	329.0	221	429.8
−3	26.6	54	129.2	110	230.0	166	330.8	222	431.6
−2	28.4	55	131.0	111	231.8	167	332.6	223	433.4
−1	30.2	56	132.8	112	233.6	168	334.4	224	435.2
0	32.0	57	134.6	113	235.4	169	336.2	225	437.0
+1	33.8	58	136.4	114	237.2	170	338.0	226	438.8
2	35.6	59	138.2	115	239.0	171	339.8	227	440.6
3	37.4	60	140.0	116	240.8	172	341.6	228	442.4
4	39.2	61	141.8	117	242.6	173	343.4	229	444.2
5	41.0	62	143.6	118	244.4	174	345.2	230	446.0
6	42.8	63	145.4	119	246.2	175	347.0	231	447.8
7	44.6	64	147.2	120	248.0	176	348.8	232	449.6
8	46.4	65	149.0	121	249.8	177	350.6	233	451.4
9	48.2	66	150.8	122	251.6	178	352.4	234	453.2
10	50.0	67	152.6	123	253.4	179	354.2	235	455.0
11	51.8	68	154.4	124	255.2	180	356.0	236	456.8
12	53.6	69	156.2	125	257.0	181	357.8	237	458.6
13	55.4	70	158.0	126	258.8	182	359.6	238	460.4
14	57.2	71	159.8	127	260.6	183	361.4	239	462.2
15	59.0	72	161.6	128	262.4	184	363.2	240	464.0
16	60.8	73	163.4	129	264.2	185	365.0	241	465.8
17	62.6	74	165.2	130	266.0	186	366.8	242	467.6
18	64.4	75	167.0	131	267.8	187	368.6	243	469.4
19	66.2	76	168.8	132	269.6	188	370.4	244	471.2
20	68.0	77	170.6	133	271.4	189	372.2	245	473.0
21	69.8	78	172.4	134	273.2	190	374.0	246	474.8
22	71.6	79	174.2	135	275.0	191	375.8	247	476.6
23	73.4	80	176.0	136	276.8	192	377.6	248	478.4
24	75.2	81	177.8	137	278.6	193	379.4	249	480.2
25	77.0	82	179.6	138	280.4	194	381.2	250	482.0
26	78.8								

To convert degrees Centigrade to degrees Fahrenheit, multiply by $\frac{9}{5}$ and add 32.
To convert degrees Fahrenheit to degrees Centigrade, subtract 32 and multiply by $\frac{5}{9}$

BIBLIOGRAPHY.

TEXT-BOOKS AND MONOGRAPHS.

American Public Health Association: Standard Methods for the Examination of Water and Sewage. 4th edition, 1920, p. 94.

Arneth, Prof.: Die Neutrophilen weissen Blutkörperchen bei Infections-Krankheiten. Fischer, Jena, 1904.

Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R.: Acute Lobar Pneumonia. Prevention and Serum Treatment. Monograph No. 7. Rockefeller Institute for Medical Research, 1917. New York.

Barker, L. F.: Monographic Medicine. Appleton & Co., New York and London, 1916.

Bass, C. C., and Johns, F. M.: Alveolodental Pyorrhea. W. B. Saunders Co., Philadelphia, 1915.

Boothby, W. M., and Sandiford, Irene: Laboratory Manual of the Technic of Basal Metabolic Rate Determination. W. B. Saunders Co., Philadelphia, 1920.

Brown, J. H.: The Use of Blood Agar for the Study of Streptococci. Monograph No. 9, Rockefeller Institute for Medical Research, 1919, New York.

Cabot, R. C.: Aplastic Anemia. Osler's Modern Medicine, **4**, 637. Lea & Febiger, Philadelphia, 1908.

Cabot, Richard C.: Clinical Examination of the Blood. Fifth edition. Wm. Wood & Co , New York, 1904.

Cabot, R. C.: Leukemia. Osler's Modern Medicine, **4**, 668. Lea & Febiger, Philadelphia, 1908.

Chamot, E. M.: Elementary Chemical Microscopy. John Wiley & Sons, Inc., New York, 1916.

Christian, Henry A.: Purpura and Allied Conditions. Oxford Medicine, **2**, 779.

Craig, C. F.: The Wassermann Test. Mosby Co., St. Louis, 1918.

Craig, C. F.: The Malarial Fevers. Wood & Co., New York, 1919.

Craig, F. A.: A Study of the Blood in Pulmonary Tuberculosis. Fourth Annual Report, Henry Phipps Institute for the Study, Treatment, and Prevention of Tuberculosis. 1909, p. 108.

Dock, G., and Bass, C. C.: Hookworm Disease, p. 178. Mosby Co., 1910.

Drinker, Cecil K.: Diseases of the Blood. The Pathological Physiology of Blood Cell Formation and Blood Cell Destruction. Oxford Medicine, **2**, 509.

Ehrlich, P.: Anemia: Histology of the Blood, Normal and Pathologic. Nothnagel's Practice of Medicine. W. B. Saunders Co., 1905.

Emerson, C. P.: Clinical Diagnosis. Lippincott Co., Philadelphia.

Ewing, James: Clinical Pathology of the Blood. Lea Brothers & Co., Philadelphia, 1901.

Fantham, H. B., Stephens, J. W. W., and Theobold, F. V.: The Animal Parasites of Man. Wood & Co., New York, 1916.

Farrington and Woll: Testing Milk and its Products. 24th edition. Mendota Book Co., 1918.

Fitz, R.: Polycythemia. Oxford Medicine, **2**, 763.

Folin, Otto: Determination of Acidity of Urine. Laboratory Manual of Biological Chemistry, p. 127, 2d edition. Appleton & Co., New York, 1919.

Gay, F. P.: Typhoid Fever: Considered as a Problem of Scientific Medicine. The Macmillan Co., New York, 1918.

Gruner, O. C.: The Biology of the Blood Cells. Wm. Wood & Co., New York, 1914.

Gulland, G. Lovell, and Goodall, Alexander: The Blood. Second edition. E. B. Treat & Co., New York, 1915.

Hand-book of Chemistry and Physics. Chemical Rubber Co., Cleveland, 1919.

Hawk, P. B.: Practical Physiological Chemistry. P. Blakiston's Son & Co., Philadelphia.

Hayem, G.: Du Sang et de ses Alterations Anatomiques. Masson et Cie, Paris, 1889.

Hensel, O., Weil, R., and Jelliffe, S. E.: The Urine and Feces in Diagnosis. p. 271. Lea Brothers & Co., Philadelphia, 1905.

Hiss, P. H., and Zinsser, H.: A Text-book of Bacteriology. Appleton & Co., New York, 1916.

Hoffman, Prof., and Schmidt, P.: (Quoted by Oliver, Lead Poisoning, p. 125). Hoeber, New York, 1914.

Karsner, H. T., and Ecker, E. E.: Principles of Immunology. J. B. Lippincott Co., Philadelphia, 1921.

Kendall, A. I.: Bacteriology, General, Pathological and Intestinal. Lea & Febiger, Philadelphia, 1921.
Kolmer, J. A.: Infection, Immunity and Specific Therapy. 2d edition. W. B. Saunders Co., Philadelphia, 1917.
Levinson, A.: Cerebrospinal Fluid in Health and Disease. Mosby Co., St. Louis, 1919.
McLean, Jay: Hemophilia. Oxford Medicine, **2**, 799.
MacCallum, W. G.: Text-book of Pathology, p. 761. W. B. Saunders Co., Philadelphia, 1916.
MacLeod, J. J. R.: Physiology and Biochemistry in Modern Medicine, p. 519. Mosby Co., St. Louis, 1918.
Mallory, F. B., and Wright, J. H.: Pathological Technique. W. B. Saunders Co., Philadelphia, 1918.
Minot, George R.: Diseases of the Blood. Clinical Discussion of the Anemias. Oxford Medicine, **2**, 589.
Myers, Victor C.: Practical Chemical Analysis of Blood. C. V. Mosby Co., St. Louis, 1921.
Noguchi, H.: Serum Diagnosis of Syphilis and the Butyric Acid Test for Syphilis. 2d edition. J. B. Lippincott Co., Philadelphia, 1911.
Nuttall, G. H. F., and Graham-Smith, G. S.: The Bacteriology of Diphtheria. University Press, Cambridge, 1913.
Ordway, T. O., and Gorham, L. W.: Leukemia. Oxford Medicine, **2**, 681.
Oxford Medicine, Volume II. Oxford University Press. (Especially the following articles: The Pathological Physiology of Blood-cell Formation and Blood-cell Destruction, by Cecil K. Drinker; Clinical Discussion of the Anemias, by George R. Minot; Leukemia, by Thomas Ordway and L. Whittington Gorham; Polycythemia, by Reginald Fitz; Purpura and Allied Conditions, by Henry A. Christian; Hemophilia, by Jay McLean.)
Park, W. H., and Williams, A.: Pathogenic Microörganisms. Lea & Febiger, Philadelphia, 1920.
Pearce, H. M., Krumbhaar, E. B., and Frazier, C. H.: The Spleen and Anemia Experimental and Clinical Studies. J. B. Lippincott Co., Philadelphia, 1918.
Pottenger, F. M.: Clinical Tuberculosis, **1**, 560. Mosby Co., St. Louis, 1917.
Rieder, Herman, and Delépine, A. S.: Atlas of Urinary Sediments. Charles Griffin & Co., London, 1899.
Schleip, Karl: Hematological Atlas. The Rebman Co., New York, 1920.
Schmidt, Ad., and Strasburger, J.: Die Faeces des Menschen. A. Hirschwald, Berlin, 1905.
Sellards, A. W.: The Principles of Acidosis and Clinical Methods for its Study. Harvard Univ. Press, Cambridge, 1919.
Simon, C. E.: A Manual of Clinical Diagnosis by Means of Laboratory Methods. Lea & Febiger, Philadelphia, 1914.
Simon, C. E.: Human Infection Carriers: Their Significance, Recognition and Management. Lea & Febiger, Philadelphia, 1919.
Stitt, E. R.: Practical Bacteriology, Blood Work and Animal Parasitology. P. Blakiston's Son & Co., Philadelphia, 1916.
Sutton, W. L.: Volumetric Analysis. 9th edition. P. Blakiston's Son & Co., Philadelphia, 1904.
Todd: Clinical Diagnosis. Fourth edition. W. B. Saunders Co., Philadelphia, 1918.
Tyson, James: The Practice of Medicine. Fourth edition. P. Blakiston's Son & Co. Philadelphia, 1906. (Especially the section on Animal Parasites.)
Von Jaksch, Rudolph: Clinical Diagnosis. Fifth English edition. Edited by A. E. Garrod. Charles Griffin & Co., London, 1905.
War Manual No. 6: Laboratory Methods of the United States Army, p. 119. Lea & Febiger, Philadelphia, 1918.
Ward, G. R.: Bedside Hematology. W. B. Saunders Co., Philadelphia, 1914.
Webster, R. W.: Diagnostic Methods, Chemical, Bacteriological and Microscopical. P. Blakiston's Son & Co., Philadelphia, 1920.
Wood, F. C.: Chemical and Microscopical Diagnosis. Appleton & Co., New York, 1917.
Wood, F. C., Vogel, K. M., and Famulener, L. W.: Laboratory Technic. Daugherty, New York, 1917.
Zinsser, H.: Infection and Resistance, p. 208. The Macmillan Co., New York, 1914.
Zinsser, Hans, Hopkins, J. K., and Ottenburg, R.: Laboratory Course in Serum Study. The Macmillan Co., New York, 1916.

ORIGINAL ARTICLES

Appleton, V. B.: Determination of Hemoglobin during Infancy by the Palmer and Van Slyke Methods. Jour. Biol. Chem., 1918, **24**, 369.

Ashby, W.: The Determination of the Length of Life of Transfused Blood Corpuscles in Man. Jour. Exp. Med., 1919, **29**, 267.

Austin, J. H., and Van Slyke, D. D.: Determination of Chlorides in Whole Blood. Jour. Biol. Chem., 1920, **41**, 345.

Avery, O. T.: A Selective Medium for B. influenzæ. Oleate-hemoglobin Agar. Jour. Am. Med. Assn., 1918, **71**, 2050.

Avery, O. T.: Determination of Types of Pneumococcus in Lobar Pneumonia. Jour. Am. Med. Assn., 1918, **70**, 17.

Barker, L. F.: On the Presence of Iron in the Granules of the Eosinophile-leukocytes, Johns Hopkins Hosp. Bull., 1894, **5**, 93.

Bass, C. C., and Johns, F. M.: The Cultivation of Malarial Plasmodia (Plasmodium Vivax and Plasmodium Falciparum) in Vitro. Jour. Exper. Med., 1912, **16**, 567.

Bass, C. C., and Johns, F. M.: A Method of Concentrating Malarial Plasmodia for Diagnostic and Other Purposes. Am. Jour. Trop. Diseases, 1915, **3**, 298.

Benedict, A. L.: Indicators in Gastric Analysis, with Special Reference to Tropeolin 00. Med. News, 1904, **84**, 597.

Benedict, A. L.: Correspondence. Cleveland Med. Jour., 1918, **17**, 279.

Benedict, S. R.: Studies in Creatine and Creatinine Metabolism. I. The Preparation of Creatine and Creatinine from Urine. Jour. Biol. Chem., 1914, **18**, 184.

Benedict, S. R., and Hitchcock, E. H.: On the Colorimetric Estimation of Uric Acid in Urine. Jour. Biol. Chem., 1915, **20**, 619.

Blankenhorn, M. A.: The Bile Content of the Blood in Pernicious Anemia. Arch. Int. Med., 1917, **19**, 344.

Blankenhorn, M. A.: The Distribution of Bile in Certain Types of Jaundice. Arch. Int. Med., 1918, **21**, 282.

Boas, H.: Die Wassermann'sche Reaktion mit bes. Berücksichtigung ihrer klinischen Verwertbarkeit. (Quoted in Special Report Series, No. 21, Med. Research Com. London, 1918, p. 6).

Bock, J. C., and Benedict, S. R.: A New Form of Colorimeter. Jour. Biol. Chem., 1918, **35**, 227.

Boggs, T. R., and Pincoffs, M. C.: A Case of Pulmonary Moniliasis in the United States. Johns Hopkins Hosp. Bull., 1915, **26**, 407.

Briggs, L. H.: Clinical Value of the Arneth Method of Blood Examination. California State Jour. Med., 1912, **10**, 337.

Brown, L., and Petroff, S. A.: The Clinical Value of Complement-fixation in Pulmonary Tuberculosis Based on a Study of 540 Cases. Am. Rev. Tuberc., 1918, **2**, 253.

Buckman, T. E., and Hallisey, J. E.: Studies in the Properties of Blood Platelets. A New Method for Counting Platelets. Jour. Am. Med. Assn., 1921, **76**, 427.

Bushnell, G. E., and Treuholtz, C. A.: Arneth's Method in the Clinical Study of Pulmonary Tuberculosis. Med. Rec., 1908, **73**, 471.

Cabot, R. C.: Ring Bodies (Nuclear Remnants?) in Anemic Blood. Jour. Med. Res., 1903, **9**, 15.

Capps, J. A.: A Study of Volume Index. Observations upon the Volume of Erythrocytes in Various Disease Conditions. Jour. Med. Res., 1903, **10**, 367.

Chace, A. F., and Myers, V. C.: The Value of Recent Laboratory Tests in the Diagnosis and Treatment of Nephritis, with Special Reference to the Chemical Examination of the Blood. Jour. Am. Med. Assn., 1916, **67**, 929.

Chace, A. F., and Myers, V. C.: Acidosis in Nephritis. Jour. Am. Med. Assn., 1920, **74**, 641.

Chatard, J. A.: The Leukocytes in Acute Lobar Pneumonia. Johns Hopkins Hosp. Rep., 1910, **15**, 89.

Chauffard, A.: (Quoted by Pearce) Pathogenie de l'ictere congenital de l'adulte. Semaine Méd., 1907, **27**, 25.

Clark, W. M.: The "Reaction" of Bacteriologic Culture Media. Jour. Infec. Dis., 1915, **17**, 109.

Clark, W. M., and Lubs, H. A.: The Colorimetric Determination of Hydrogen-ion Concentration and its Applications in Bacteriology. Jour. Bacteriol., 1917, **2**, 1, 109 and 191.

Cole, S. W., and Onslow, R.: On a Substitute for Peptone and a Standard Nutrient Medium for Bacteriological Purposes. Lancet, 1916, **2**, 9 and 1011.

Cowdry, E. V.: The Staining of Mitochondria in Human Lymphocytes with Janus Green. Anat. Rec., 1914, **8**, 140.

Craig, C. F.: The Relation of Certain Bacteria to Non-specific Reactions with the Complement-fixation Test for Lues. Jour. Exp. Med., 1911, **13**, 521.

Craig, C. F.: The Results and Interpretation of the Wassermann Test. Am. Jour. Med. Sci., 1915, **149**, 41.

Craig, C. F.: Observations upon Complement-fixation in the Diagnosis of Pulmonary Tuberculosis. Am. Jour. Med. Sci., 1915, **150**, 782.

Craig, C. F., and Nichols, H. J.: The Effect of the Ingestion of Alcohol on the Result of the Complement-fixation Test in Syphilis. Jour. Am. Med. Assn., 1914, **57**, 474.

Cummer, C. L.: Device for Withdrawing Blood from Veins. Jour. Lab. and Clin. Med., 1919–20, **5**, 257.

Daland, J.: A New Hematokrit and a New Technique. Tr. Coll. Phys. Philadelphia. 1894, **16**, 192.

Dexter, R., and Cummer, C. L.: The Occurrence of Native Antisheep Amboceptor in Human Serum and its Importance in the Performance of the Wassermann Reaction. Arch. Int. Med., 1912, **9**, 605.

Dexter, R., and Cummer, C. L.: The Importance of the Early Diagnosis of Syphilis. Jour. Am. Med. Assn., 1912, **59**, 1254.

Dobell, C.: On the Nomenclature of the Spirochete of Syphilis. Medical Research Committee. Special Report, No. 19, p. 47. London, 1918.

Dock, G.: Methods, Value and Limitations of the Knowledge of the Gastric Contents. Jour. Am. Med. Assn., 1905, **45**, 1385.

Döhle, Prof. Dr.: Leukocyteneinschlusse bei Scharlach. Centralbl. f. Bact., 1 Abt. Orig., 1911, **61**, 63.

Dreyer, G, and Inman, A. C.: The Agglutination Curve and its Importance in the Diagnosis of Typhoid and Paratyphoid Fevers in Inoculated Persons. Lancet, 1817, **1**, 363.

Drinker, C. K., and Hurwitz, S. H.: The Factors of Coagulation in Primary Pernicious Anemia. Arch. Int. Med., 1915, **15**, 733.

Duke, W. W.: The Relation of Blood Platelets to Hemorrhagic Disease. Jour. Am. Med. Assn., 1910, **55**, 1185.

Ecker, E. E.: An Apparently Practical Method for the Isolation of Pfeiffer's Bacillus from Sputum. Jour. Am. Med. Assn., 1918, **71**, 1482.

Ecker, E. E., and Sasano, K.: A Rapid Method for Preparing Antigens from Normal Heart Muscle. Jour. Infec. Dis., 1919, **25**, 174.

Einhorn, M., and Rosenbloom, J.: A Study of the Duodenal Contents in Man. Arch. Int. Med., 1910, **6**, 666.

Einhorn, M.: A Practical Method of Obtaining the Duodenal Contents in Man. Med. Rec. 1910, **77**, 98.

Einhorn, M.: Historical Sketch of the Development of the Duodenal Tube. Am. Jour. Med. Sci., 1916, **151**, 202.

Fildes, P., and McIntosh, J.: The Wassermann Reaction and its Application to Neurology. Brain, 1913–14, **36**, 193.

Folin, Otto: Approximately Complete Analysis of Thirty "Normal" Urines. Am. Jour. Physiol., 1905, **13**, 45.

Folin, Otto, and Denis, W.: New Methods for the Determination of Total Non-protein Nitrogen, Urea and Ammonia in Blood. Jour. Biol. Chem., 1912, **11**, 527.

Folin, Otto, and Denis, W.: On the Colorimetric Determination of Uric Acid in Urine. Jour. Biol. Chem., 1913, **14**, 95.

Folin, Otto, and Denis, W.: On the Creatinine and Creatine Content of the Blood. Jour. Biol. Chem., 1914, **17**, 487.

Folin, Otto, and Denis, W.: Nitrogen Determination by Direct Nesslerization. The Use of the Colorimeter. Jour. Biol. Chem., 1916, **26**, 484.

Folin, Otto, and Doisy, E. A.: Impure Picric Acid as a Source of Error in Creatine and Creatinine Determination. Jour. Biol. Chem., 1916–17, **28**, 349.

Folin, O., and Farmer, C. J.: A New Method for the Determination of Total Nitrogen in Urine. Jour. Biol. Chem., 1912, **11**, 493.

Folin, O., and MacCallum, A. B.: A New Method for the (Colorimetric) Determination of Uric Acid in Urine. Jour. Biol. Chem., 1913, **13**, 363.

Folin, O., and Morris, J. L.: On the Determination of Creatinine and Creatine in Urine. Jour. Biol. Chem., 1914, **17**, 469.

Folin, O., and Wu, H.: A System of Blood Analysis. Jour. Biol. Chem., 1919, **38**, 81.

Folin, O., and Wu, H.: A System of Blood Analysis "New Method for Determination of Sugar." Jour. Biol. Chem., 1919, **38**, 106.

Folin, O., and Wu, H.: A System of Blood Analysis. Supplement 1. A Simplified and Improved Method for Determination of Sugar. Jour. Biol. Chem., 1920, **41**, 367.

Friedenwald, J., and Kiefer, R. F.: On the Value of the Quantitative Estimation of Dissolved Albumin in the Gastric Contents in the Diagnosis of Cancer of the Stomach. Am. Jour. Med. Sci., 1916, **152**, 321.

Galt, H. M., and Iles, C. C.: A Study of the Boas-Oppler Bacillus. Jour. Path. and Bact., 1914-15, **19**, 239.

Gibson, C. L.: The Value of the Differential Leukocyte Count in Acute Surgical Diseases. Ann Surg., 1906, **43**, 485.

Giffin, H. Z., and Sanford, A. H.: Clinical Observations Concerning the Fragility of Erythrocytes. Jour. Lab. and Clin. Med., 1918-19, **4**, 465.

Graham, G. S.: The Oxidizing Ferment of the Myelocyte Series of Cells and its Demonstration by an Alphanaphthol-pyronin Method. Jour. Med. Res., 1916, **35**, 231.

Graham, G. S.: Benzidin as a Peroxidase Reagent for Blood Smears and Tissues. Jour. Med. Res., 1918, **39**, 15.

Gray, Horace: Cell-counting Technic: A Study of Priority. Am. Jour. Med. Sci., 1921, **162**, 526.

Greenwald, I.: The Estimation of Non-protein Nitrogen in Blood. Jour. Biol. Chem., 1918, **34**, 97.

Hamman, L., and Hirschmann, I. I.: Studies on Blood Sugar. Alimentary Hyperglycemia and Glycosuria as a Test of Sugar Tolerance. Arch. Int. Med., 1917, **20**, 761.

Harrington, T. F.: Industrial Benzol Poisoning in Massachusetts. Boston Med. and Surg. Jour., 1917, **177**, 203.

Harvey, S. C.: The Quantitative Determination of the Chlorides in the Urine. Arch. Int. Med., 1910, **6**, 12.

Hess, A. F.: A Method of Obtaining Cultures from the Duodenum of Infants. Jour. Infect. Dis., 1912, **11**, 71.

Hill, L. W.: Report on Leukocytic Inclusion Bodies. Boston Med. and Surg. Jour., 1914, **170**, 792.

Hopkins, J. G.: A Method for Standardizing Bacterial Vaccines. Jour. Am. Med. Assn., 1913, **60**, 1615.

Howell, W. H.: The Life History of the Formed Elements of the Blood, Especially the Red Blood Corpuscle. Jour. Morphol., 1890, **4**, 57.

Howell, W. H.: The Condition of the Blood in Hemophilia, Thrombosis and Purpura. Arch. Int. Med., 1914, **13**, 76.

Howland, J., and Marriott, W. McK.: Acidosis Occurring with Diarrhea. Am. Jour. Dis. Child., 1916, **11**, 309.

Hurwitz, S. H., and Lucas, W. P.: A Study of the Blood in Hemophilia. Arch. Int. Med., 1916, **17**, 543.

Hurwitz, S. H., Meyer, K. F., and Ostenberg, Z.: On a Colorimetric Method of Adjusting Bacteriologic Media to any Optimum Hydrogen-ion Concentration. Proc. Soc. Exper. Biol. and Med., 1915, **13**, 24.

Hurwitz, S. H., Meyer, K. F., and Ostenberg, Z.: A Colorimetric Method for the Determination of the Hydrogen-ion Concentration of Biological Fluids, with Special Reference to the Adjustment of Bacteriological Culture Media. Johns Hopkins Hosp. Bull., 1916, **27**, 16.

Jansky: Isohemagglutination: Recommendation that the Jansky Classification be Adopted for Universal Use. Sborn. Klin., 1907, **8**, 85. Quoted in Jour. Am. Med. Assn., 1921, **78**, 130.

Jenner, L.: A New Preparation for Rapidly Fixing and Staining Blood. Lancet, 1899, **1**, 370.

Johnson, F. B.: Filarial Infection—An Investigation of its Prevalence in Charleston, S. C. South. Med. Jour., 1915, **8**, 630.

Jousset, A.: Sur une nouvelle méthode de recherche du bacille tuberculeux. Bull et mem. Soc. Med. des Hôp. de Paris, 1903, **20**, 23. Also Semaine Méd., 1903, **22**, 22.

Karsner, H. T.: Transfusion with Tested Bloods. Jour. Am. Med. Assn., 1918, **70**, 769.

Karsner, H. T.: and Koeckert, H. L.: The Influence of Desiccation on Human Normal Isohemagglutinins. Jour. Am. Med. Assn., 1919, **73**, 1207.

Kelly, T. H.: Detection of Small Amounts of Blood. Jour. Lab. and Clin. Med., 1915-16, **1**, 897.

Kober, P. A.: An Improved Nephelometer-colorimeter. Jour. Biol. Chem., 1917, **29**, 155.

Kolmer, J. A.: Leukocytic "Inclusion Bodies" with Special Reference to Scarlet Fever. Am. Jour. Dis. Child., 1912, **4**, 1.

Lambert, S. W., and Patterson, H. S.: Poisoning by Mercuric Chloride and its Treatment. Arch. Int. Med., 1915, **16**, 865.

Larrabee, R. C.: The Volume Index of the Red Corpuscles. Jour. Med. Res., 1911, **24**, 15.

Lawson, M. R.: Free Malarial Parasites and the Effect of the Migration of the Parasites of Tertian Malarial Infections. Jour. Exp. Med., 1914, **19**, 523.

Lee, R. I.: A Simple and Rapid Method for the Selection of Suitable Donors for Transfusion by the Determination of Blood Groups. British Med. Jour., 1917, **2**, 684.

Lee, R. I., Minot, G. R., and Vincent, B.: Splenectomy in Pernicious Anemia. Jour. Am. Med. Assn., 1916, **67**, 719.

Lee, R. I., and Vincent, B.: The Coagulation of Normal Human Blood. Arch. Int. Med., 1914, **13**, 398.

Lee, R. I., and White, P. D.: A Clinical Study of the Coagulation Time of Blood. Am. Jour. Med. Sc., 1913, **145**, 495.

Levine, Max: Notes on Experiences with the Presumptive and Confirmatory Tests for B. coli in Water Analysis for the American Expeditionary Forces. Jour. Am. Water Works Assn., 1920, **7**, 188.

Levine, Max: Dysentery and Allied Bacilli. Jour. Infect. Dis., 1920, **27**, 31.

Levy, R. L., Rowntree, L. G., and Marriott, W. McK.: A Simple Method for Determining Variations in the Hydrogen-ion Concentration of the Blood. Arch. Int. Med., 1915, **16**, 389.

Lewis, R. C., and Benedict, S. R.: A Method for the Estimation of Sugar in Small Quantities of Blood. Jour. Biol. Chem., 1915, **10**, 61.

Libman, E.: Clinical Significance of Blood Examinations: The Bacteriology. Internat. Clin., 1919, 29 S., **3**, 125.

Lord, F. T., Scott, A. C., and Nye, R. N.: Relation of Influenza Bacillus to the Recent Epidemic of Influenza. Jour. Am. Med. Assn., 1919, **72**, 188.

Lucas, W. P.: The Value of the Blood Picture in the Early Diagnosis of Measles, Especially in Relation to the Question of Isolation. Am. Jour. Dis. Child., 1914, **7**, 149.

Lundsgaard, C.: I. Studies of Oxygen in the Venous Blood. II. Studies of the Oxygen Unsaturation in the Venous Blood of a Group of Patients with Circulatory Disturbances. Jour. Exp. Med., 1918, **27**, 179. III. Determinations on Five Patients with Compensated Circulatory Disturbances. Jour. Exp. Med., 1918, **27**, 199. IV. Determinations on Five Patients with Incompensated Circulatory Disturbances. Jour. Exp. Med., 1918, **27**, 219.

Lyon, B. B. V.: Diagnosis and Treatment of Diseases of the Gall-bladder and Bile Ducts. Jour. Am. Med Assn., 1919, **73**, 980.

McLean, F. C.: Clinical Determination of the Renal Function by an Index of Urea Excretion. Jour. Am. Med. Assn., 1915, **66**, 415.

McLean, F. C.: The Numerical Laws Governing the Rate of Excretion of Urea and Chlorides in Man. I. An Index of Urea Excretion and the Normal Excretion of Urea and Chlorides. Jour. Exp. Med., 1915, **22**, 212 and 366.

McNeil, H. L.: The Use of the Duodenal Catheter in Diagnosis. Am. Jour. Med. Sci., 1916, **151**, 106.

MacNeal, W. J., and Chace, A. F.: A Contribution to the Bacteriology of the Duodenum. Arch. Int. Med., 1913, **12**, 178.

MacRobert, R. G.: The Cause of Lumbar Puncture Headache. Jour. Am. Med. Assn., 1918, **70**, 1350.

Marriott, W. McK.: The Determination of Alveolar Carbon Dioxid Tension by a Simple Method. Jour. Am. Med. Assn., 1916, **66**, 1594.

Marriott, W. McK.: A Method for the Determination of the Alkali Reserve of the Blood Plasma. Arch. Int. Med., 1916, **17**, 840.

Marshall, E. K.: A Rapid Clinical Method for the Estimation of Urea in Urine. Jour. Biol. Chem., 1913, **14**, 283.

Marshall, E. K.: A New Method for the Determination of Urea in Blood. Jour. Biol. Chem., 1913, **15**, 487.

Marshall, E. K.: The Determination of Urea in Urine (Second Communication). Jour. Biol. Chem., 1913, **15**, 495.

Meader, C. N.: Five Cases of Relapsing Fever Originating in Colorado, with Positive Blood Findings in Two. Colorado Med., 1915, **12**, 365.

Meltzer, S. J.: The Disturbance of the Law of Contrary Innervation as a Pathogenetic Factor in the Diseases of the Bile Ducts and Gall-bladder. Am. Jour. Med. Sci., 1917, **153**, 469.

Miller, H. R.: A Review of the Complement-fixation Test in Tuberculosis. Jour. Lab. and Clin. Med., 1915-16, **1**, 816.

Miller, H. R., and Zinsser, H.: Complement-fixation Test in Tuberculosis. Proc. Soc. Exp. Biol. and Med., 1916, **13**, 134.

Miller, J. A., and Reed, M. A.: Studies of the Leukocytes in Pulmonary Tuberculosis and Pneumonia. Arch. Int. Med., 1912, **9**, 609.

Miller, S. R.: The Normal Differential Leukocyte Count. Johns Hopkins Hosp. Bull., 1914, **25**, 317.

Miller, S. R., and Baetjer, W. A.: Bence-Jones-Proteinuria. Some Observations on its Occurrence, with Particular Reference to Nephritis and Hypertension. Jour. Am. Med. Assn., 1918, **70**, 137.

Miller, S. R., Brush, N. D., Hammers, J. S., and Felton, L. D.: A Further Study of the Diagnostic Value of the Colloidal-gold Reaction, Together with a Method for the Preparation of the Reagent. Johns Hopkins Hosp. Bull., 1915, **26**, 391.

Minor, C. L., and Ringer, P. H.: Arneth's Method of Blood Counting, its Prognostic Value in Pulmonary Tuberculosis. Am. Jour. Med. Sci., 1911, **141**, 638.

Minot, G. R.: Diminished Blood Platelets and Marrow Insufficiency. A Classification and Differential Diagnosis of Purpura Hemorrhagica, Aplastic Anemia, and Allied Conditions. Arch. Int. Med., 1917, **19**, 1062.

Minot, G. R., and Denny, G. P.: Prothrombin and Antithrombin Factors in the Coagulation of Blood. Arch. Int. Med., 1916, **17**, 101.

Minot, G. R., and Lee, R. I.: The Blood Platelets in Hemophilia. Arch. Int. Med., 1916, **18**, 474.

Montgomery, F. H., and Ormsby, O. S.: Systemic Blastomycosis, its Etiology, Pathologic and Clinical Features; the Relation of Blastomycosis to Coccidioidal Granuloma. Arch. Int. Med., 1908, **2**, 1.

Morris, R. S.: Note on the Occurrence of Howell's Nuclear Particles in Experimental Anemia of the Rabbit and in Human Blood. Johns Hopkins Hosp. Bull., 1907, **18**, 198.

Morris, R. S.: The Value of Ehrlich's Triacid Stain in Blood Work. Jour. Am. Med. Assn., 1910, **55**, 501.

Mosenthal, H. O.: Renal Function as Measured by the Elimination of Fluids, Salt and Nitrogen, and the Specific Gravity of the Urine. Arch. Int. Med., 1915, **16**, 733.

Mosenthal, H. O., and Lewis, D. S.: A Comparative Study of Tests for Renal Function. Jour. Am. Med. Assn., 1916, **67**, 933.

Moss, W. L.: Studies on Isoagglutinins and Isohemolysins. Johns Hopkins Hosp. Bull., 1910, **21**, 63.

Murphy, J. B., and Ellis, A. W. M.: Experiments on the Role of Lymphoid Tissue in the Resistance to Experimental Tuberculosis in Mice. Jour. Exp. Med., 1914, **20**, 397.

Musgrave, P.: Cyto-diagnosis: A Study of the Cellular Elements in Serous Effusions. Boston Med. and Surg. Jour., 1903, **148**, 256.

Musgrave, P.: Examination of Pleural Fluids with Reference to Their Etiology and Diagnostic Value. Boston Med. and Surg. Jour., 1904, **151**, 317.

Musser, J. H.: Study of a Case of Aplastic Anemia. Arch. Int. Med., 1914, **14**, 275.

Myers, V. C.: Chemical Changes in the Blood in Disease. Post-Graduate, 1914, **29**, 505; and Jour. Lab. and Clin. Med., 1919-20, **5**, 343 and 418.

Myers, V. C.: Simple Methods for the Determination of Nitrogenous Constituents in Urine. Post-Graduate, 1914, **29**, 737.

Myers, V. C.: Determination of Blood Urea. Post-Graduate, 1914, **29**, 505.

Myers, V. C.: Chemical Constitution of the Blood in Health and Disease. VI. Total Solids, Total Nitrogen and Chlorides. Post-Graduate, 1915, **30**, 35.

Myers, V. C.: A Simple Colorimeter for Clinical Purposes. Jour. Lab. and Clin. Med., 1915-16, **1**, 760.

Myers, V. C., and Bailey, C. V.: The Lewis and Benedict Method for the Estimation of Blood Sugar, with Some Observations Obtained in Disease. Jour. Biol. Chem., 1916, **24**, 147.

Myers, V. C., and Fine, M. C.: Essentials of Pathological Chemistry, Including Description of the Chemical Methods Employed in Medical Diagnosis. Reprinted from Post-Graduate, 1913.

Myers, V. C., and Killian, J. A.: Studies on Animal Diastases. I. The Increased Activity of the Blood in Diabetes and Nephritis. Jour. Biol. Chem., 1917, **29**, 179.

Myers, V. C., and Lough, W. G.: The Creatinin of the Blood in Nephritis, its Diagnostic Value. Arch. Int. Med., 1915, **16**, 536.

Myers, V. C., and Short, J. J.: The Estimation of Chlorides in Blood. Jour. Biol. Chem., 1920, **44**, 47.

Nicoll, M.: Present-day Opinions of the Value of the So-called Inclusion Bodies in Scarlet Fever. Arch. Pediat., 1913, **30**, 346.

Opie, E. L.: The Part of Enzymes in Tuberculous Lesions. Proc. 6th Internat. Cong. Tuberc., 1908, Part I, p. 255.

Palmer, W. W., and Van Slyke, D.D.: Studies of Acidosis. IX. Relationship between Alkali Retention and Alkali Reserve in Normal and Pathological Individuals. Jour. Biol. Chem., 1917, **32**, 499.

Palmer, W. W.: The Colorimetric Determination of Hemoglobin. Jour. Biol. Chem., 1918, **33**, 119.

Pepper, O. H. P., and Peet, M. M. The Resistance of Reticulated Erythrocytes Arch. Int. Med., 1913, **12**, 81.

Petroff, S. A.: Some Cultural Studies on the Tubercle Bacillus. Johns Hopkins Hosp. Bull., 1915, **26**, 276.

Petroff, S., A: Serological Studies of Tuberculosis. Am. Rev. Tuberc., 1917–18, **1**, 36.

Poulton, E. P.: The Significance of Alveolar Carbon Dioxide Determinations in the Treatment and Prognosis of Diabetes. British Med. Jour., 1915, **2**, 392.

Pratt, J. H.: History of Blood Platelets, Relation to Disease. Johns Hopkins Hosp. Bull., 1905, **16**, 201.

Pritchett, I. W., and Stillman, E. G.: The Occurrence of Bacillus Influenza in Throats and Saliva. Jour. Exp. Med., 1919, **29**, 259.

Regan, J. C.: Anatomic Points Determining the Direction of the Needle and the Proper Route for Lumbar Puncture in Children and Adults. Am. Jour. Med. Sc., 1919, **157**, 83.

Rehfuss, M. E.: A Modified Gastroduodenal Tube. New York Med. Jour., 1914, **100**, 374.

Rehfuss, M. E.: A New Method of Gastric Testing, with a Description of a Method for the Fractional Testing of the Gastric Juice. Am. Jour. Med. Sci., 1914, **147**, 848.

Rehfuss, M. E., Bergeim, O., and Hawk, P. B.: Gastro-intestinal Studies. The Fractional Study of Gastric Digestion. Jour. Am. Med. Assn., 1914, **63**, 909.

Riesman, D.: Pleural Effusion with Inversion of the Diaphragm Producing an Abdominal Tumor; Together with Remarks on Acute Pulmonary Edema Following Tapping. Am. Jour. Med. Sci., 1920, **159**, 353.

Rivas, D., and Smith, A. J.: The Early Diagnosis of Filariasis. South. Med. Jour., 1912, **5**, 631.

Roberts, D.: A Benzidin Tablet Test for Occult Blood. Jour. Am. Med. Assn., 1915. **65**, 244.

Robertson, M.: Notes upon Certain Anaërobes Isolated from Wounds. Jour. Path. and Bact., 1915–16, **20**, 327.

Robinson, A. S.: A Clinical Study of the Secretions on the Proximal and Distal Sides of the Pylorus. Arch. Int. Med., 1917, **19**, 220.

Rose, W. C.: A Modified Method for the Clinical Estimation of Pepsin. Arch. Int. Med., 1910, **5**, 459.

Rosenow, E. C.: The Newer Bacteriology of Various Infections as Determined by Special Methods. Jour. Am. Med. Assn., 1914, **63**, 903.

Rothera, A. C. H.: Note on the Sodium Nitro-prusside Reaction for Acetone. Jour. Physiol., 1908, **37**, 491.

Rous, Peyton, and Turner, J. R.: A Rapid and Simple Method of Testing Donors for Transfusion. Jour. Am. Med. Assn., 1915, **64**, 1980.

Rowntree, L. G., and Fitz, R.: Studies of Renal Function in Renal, Cardiorenal and Cardiac Diseases. Arch. Int. Med., 1913, **11**, 258.

Rowntree, L. G., and Geraghty, J. T.: An Experimental and Clinical Study of the Functional Activity of the Kidneys by means of Phenolsulphonephthalein. Jour. Pharmac. and Exp. Therap., 1910, **1**, 579.

Rowntree, L. G., and Geraghty, J. T.: The Phthalein Test. An Experimental and Clinical Study of Phenolsulphonephthalein in Relation to Renal Function in Health and Disease. Arch. Int. Med., 1912, **9**, 284.

Ruediger, E. H.: A Comparison of Six Different Antigens in the Wassermann Reaction. Jour. Infect. Dis., 1919, **24**, 31.

Ruediger, E. H.: The Influence of Antigen Dilution on the Wassermann Reaction. Jour. Infect. Dis., 1919, **25,** 224.

Russell, F. F.: The Isolation of Typhoid Bacilli from Urine and Feces with the Description of a New Double Sugar Tube Medium. Jour. Med. Res., 1911–12, **25,** 217.

Scott, R. W.: The Effect of the Accumulation of Carbon Dioxide on the Tidal Air, and on the H-ion Concentration of the Arterial Blood in the Decerebrate Cat. Am. Jour. Physiol., 1917, **44,** 196.

Selling, L.: Benzol as a Leukotoxin. Studies on the Degeneration and Regeneration of the Blood and Hematopoietic Organs. Johns Hopkins Hosp. Rep., 1916, **17,** 83.

Selling, L.: A Preliminary Report of Some Cases of Purpura Hemorrhagica Due to Benzol Poisoning. Johns Hopkins Hosp. Bull., 1910, **21,** 33.

Shawan, H. K.: The Principle of Blood Grouping Applied to Skin Grafting. Am. Jour. Med. Sc., 1919, **157,** 503.

Smith, J. W., and MacNeal, W. J.: Comparative Study of Different Antigens and of Different Temperatures of Incubation in the Wassermann Test. Jour. Infect. Dis., 1917, **21,** 233.

Smithies, F.: The Significance of Gastric Ulcer with Respect to Gastric Cancer. Jour. Am. Med. Assn., 1913, **61,** 1793.

Solis-Cohen, M., and Strickler, A.: The Leukocytic Picture in Pulmonary Tuberculosis. Am. Jour. Med. Sc., 1911, **142,** 691.

Sondern, F. E.: The Present Attitude of Blood Examination for Diagnostic Purposes. Boston Med. and Surg. Jour., 1903, **153,** 690.

Sondern, F. E.: The Present Attitude of Blood Examination in Surgical Diagnosis. Med. Rec., 1905, **67,** 452.

Stober, A. M.: Systemic Blastomycosis. A Report of Its Pathological, Bacteriological and Clinical Features. Arch. Int. Med., 1914, **13,** 509.

Suggestions to Medical Examiners by the Insurance Editor, "The Urine." Med. Rec., 1912, **82,** 449, 634, 680, and 1914, **85,** 1179.

Swift, H. F.: A Comparative Study of Serum Diagnosis in Syphilis. Arch. Int. Med., 1909, **4,** 376.

Swift, H. F., and Ellis, A. W. M.: The Cerebrospinal Fluid in Syphilis. Jour. Exp. Med., 1913, **18,** 162.

Thayer, W. S.: On Hemolytic Jaundice. Illinois Med. Jour., 1911, **19,** 174.

Thomas, B. A.: Chromocystoscopy in Functional Renal Diagnosis Based upon the Employment of Indigocarmin. Surg., Gyn. and Obst., 1909, **8,** 368.

Thomas, B. A.: The Quantitative Determination of Functional Renal Insufficiency by the Duboscq Colorimeter; Indigocarmin Versus Phenolsulphonephthalein. Am. Jour. Med. Sc., 1911, **142,** 376.

Thomas, B. A.: The Value of Chromo-ureteroscopy in Functional Kidney Diagnosis. Surg., Gyn. and Obstet., 1911, **12,** 345.

Thomas, B. A.: The Results of Two Hundred Chromo-ureteroscopies Employing Indigocarmin as a Functional Kidney Test. Jour. Am. Med. Assn., 1913, **60,** 185.

Thomas, B. A.: The Role of Functional Kidney Tests and Preoperative and Postoperative Treatment in the Reduction of Prostatectomy Mortality. Jour. Am. Med. Assn., 1914, **63,** 1909.

Thomas, B. A., and Birdsall, J. C. Comparative Results of Various Functional Kidney Tests. Jour. Am. Med. Assn., 1917, **69,** 1747.

Thomson, D.: A New Culture Medium for the Gonococcus. British Med. Jour., 1917, **1,** 869.

Tileston, W., and Griffin, W. A.: Chronic Family Jaundice. Am. Jour. Med. Sci., 1910, **139,** 847.

Van Giesen, I.: The So-called "Much Granules" in Tuberculosis. Collected Studies from the Research Laboratory of the Department of Health, New York City, 1911, **6,** 265.

Van Slyke, D. D.: Gasometric Determination of the Oxygen and Hemoglobin of Blood. Jour. Biol. Chem., 1918, **33,** 127.

Van Slyke, D. D., and Cullen, G. E.: A Permanent Preparation of Urease and its Use for Rapid and Accurate Determination of Urea. Jour. Am. Med. Assn., 1914, **62,** 1558.

Van Slyke, D. D., and Cullen G. E.: The Bicarbonate Concentration of the Blood Plasma: its Significance and its Determination as a measure of Acidosis. Jour. Biol. Chem., 1917, **30,** 289.

Van Slyke, D. D., and Donleavy, J. J.: A Simplification of the McLean-Van Slyke Method for Determination of Plasma Chlorides. Jour. Biol. Chem., 1919, **37,** 55.

Vogel, K. M.: Detection of Mercury in the Excretions. Jour. Am. Med. Assn., 1914, **62,** 532.

von Wedel, H.: A Contribution to the Study of the Complement-fixation Reaction for Tuberculosis. Jour. Immunol., 1918, **3,** 351.

Walker, O. J.: An Index of Body Resistance in Acute Inflammatory Diseases as Indicated by Examination of the Blood. Jour. Am. Med. Assn., 1919, **72,** 1453,

Walpole, G. S.: A Method of Titrating Physiological Fluids. Proc. Physiol. Soc.. London, 1910, xxvii.

Warfield, L. W.: The Differential Leukocyte Count in the Newborn. Am. Med., 1902, **4,** 457.

Waring, J. J.: Relapsing Fever Endemic in Colorado. Am. Jour. Med. Sci., 1918, **155,** 819.

Webb, G. B., and Williams, W. W.: Some Hematological Studies in Tuberculosis. Trans. Natl. Assn. for Study and Prevention of Tuberculosis, 1909, p. 231.

West, C. E.: The Bacteriology of Chronic Postnasal Catarrh. Proc. Roy. Soc. Med., 1910–11, **4,** Otol. Sec. 43.

White, C. Y., and Pepper, W.: Granular Degeneration of the Erythrocyte. Tr. Assn. Am. Phys., 1901, **16,** 410.

Williamson, C. S.: Influence of Age and Sex on Hemoglobin. A Spectrophotometric Analysis of Nine Hundred and Nineteen Cases. Arch. Int. Med., 1916, **18,** 505.

Wilson, T. M.: On the Chemistry and Staining Properties of Certain Derivatives of the Methylene Blue Group when Combined with Eosin. Jour. Exp. Med., 1907, **9,** 645.

Wilson, M. A.: A Contribution to the Study of the Complement-fixation Reaction in Tuberculosis. Jour. Immunol., 1918, **3,** 345.

Wolff, W., and Junghans, P.: Ueber die quantitative Bestimmung gelöster Eiweisstoffe im Mageninhalt. Berl. klin. Wchnschr., 1911, **48,** 978.

Wright, J. H.: The Histogenesis of the Blood Platelets. Jour. Morphol., 1910, **21,** 263.

Yaoita, S.: Ein neues Verfahren zur Auffindung spärlicher Parasiteneier in Feces. Deutsche med. Wchnschr., 1912, **38,** 1540.

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